STRUCTURE-ACTIVITY RELATIONSHIPS OF AGENTS MODIFYING CHOLINERGIC TRANSMISSION

ANNUAL REPORT

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April 1988

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Ft. Detrick, Frederick, MD 21701-5012

Contract No. DAMD17-87-C-7113

The University of Iowa
Iowa City, Iowa 52242

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SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188

1a. REPORT SECURITY CLASSIFICATION: UNCLASSIFIED	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION/AVAILABILITY OF REPORT: Approved for public release; distribution unlimited
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION: The University of Iowa; 6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION
6c. ADDRESS (City, State, and ZIP Code): Iowa City, Iowa 52242	7b. ADDRESS (City, State, and ZIP Code)
8a. NAME OF FUNDING/SPONSORING ORGANIZATION: U.S. Army Medical Research & Development Command; 8b. OFFICE SYMBOL (If applicable): SGRD-RMI-S	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER: DAMD 17-87-C-7113
8c. ADDRESS (City, State, and ZIP Code): Fort Detrick, Frederick, Maryland 21701-5012	10. SOURCE OF FUNDING NUMBERS: PROGRAM ELEMENT NO. 62734A; PROJECT NO. 3M1-62734A875; TASK NO. BB; WORK UNIT ACCESSION NO. 111

11. TITLE (Include Security Classification)

(U) Structure-Activity Relationships of Agents Modifying Cholinergic Transmission

12. PERSONAL AUTHOR(S)

J.P. Long, R.K. Bhatnagar, and J.G. Cannon

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Annual Report	FROM 4/1/87 TO 3/31/88	1988 April	59

16. SUPPLEMENTARY NOTATION

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	Hemicholiniums; Acetylcholine (ACh); Inhibitors of synthesis of ACh; Inhibitors of cholinesterase; Structure activity relationships of hemicholiniums; choline transport;
07	03		
06	15		

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

Structure activity relationship studies of hemicholinium (HC-3) analogs are directed toward better understanding both the spatial aspect of the receptor and interatomic distances between the 2 cationic heads. Several series of compounds including 4,4' biphenyl and trans/trans-cyclohexyl derivatives are being investigated and both series are potent inhibitors of acetylcholine synthesis. One compound, N-methyl 4-methyl piperidine derivative in the biphenyl series, is a very active inhibitor of ACh synthesis. The tertiary amine analog is an active inhibitor of synthesis of acetylcholine. It is much less active than the quaternary derivative. Recently we discovered that 4-hydroxy-piperidine analogs of hemicholinium have the same therapeutic index for protection of mice against paraoxan-induced toxicity as pyridostigmine. Keywords: Pharmacological antagonists. (AW)

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
☐ UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT ☐ DTIC USERS	UNCLASSIFIED
22a. NAME OF RESPONSIBLE INDIVIDUAL: Mary Frances Bostian	22b. TELEPHONE (Include Area Code): 301-663-7325; 22c. OFFICE SYMBOL: SGRD-RMI-S

DD Form 1473, JUN 86 — Previous editions are obsolete. — SECURITY CLASSIFICATION OF THIS PAGE

18. Subject Terms (continued)

Microelectrode studies

SUMMARY OF ANNUAL REPORT

The purpose of this research is synthesis and biological evaluation of analogs of hemicholinium (HC-3). These agents decrease the ability of cholinergic neurons to synthesize acetylcholine by inhibition of choline transport. Objectives of this research is to develop compounds which can neutralize excess acetylcholine within a cholinergic synapse. Two major approaches involve (1) decreasing the content of acetylcholine within a synapse or (2) desensitization of cholinergic receptors at post-synaptic sites. Another series of compounds have been defined which are potent antagonists of paraoxon-induced toxicity. These agents are weak inhibitors of cholinesterase and may be acting by protecting the esteratic site.

Another area of research which has evolved from this work is the recognition that minor alteration in structure about the bis cationic sites of the synthetic compounds can produce agents which are selective inhibitors of acetylcholinesterase. One major observation is that two compounds which are tertiary amines are selective inhibitors. The most active is only about one-tenth as active as physostigmine, but the selectivity of these compounds may be useful. Past research has demonstrated the difficulty in obtaining selective, non-quaternary amines for inhibition of acetylcholinesterase.

Detailed studies of choline transport into cholinergic neurons and a discussion of possible molecular mechanisms are included. These agents have allowed these studies and the mechanism is much more complex than indicated in the literature. Interconversion of high and low affinity states involved in choline transport appears to be a reality. Impaired choline transport may be related to various diseases which are related to deficiencies of acetylcholine.

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TABLE OF CONTENTS (continued)

I. Statement of the problem: The object of this research is the synthesis and biological evaluations of analogs of hemicholinium which are known to decrease synthesis of acetylcholine. Compounds in this series are known to antagonize choline uptake into the cholinergic neuron and inhibit nicotinic receptors. These are theoretical approaches to decrease the amount of acetylcholine within a synapse. Minor structural changes yield compounds which will inhibit acetylcholinesterase or show preference for pre- or post-junctional sites. All of the above mechanisms of action are theoretical approaches to develop compounds to antagonize organophosphate-induced toxicity.

II. Background: At the University of Iowa, Hemicholinium (HC-3) and a large number of analogs were synthesized from 1954-1969. Depending on the structure, a number of the agents were inhibitors of acetylcholinesterase and/or agents which inhibited the synthesis of acetylcholine (HC-3-like). Due to our research over the past five years, much knowledge has been added to both the chemistry and biology of these very active chemicals.

Selected acetal derivatives of HC-3 antagonized nicotinic agents (nicotine and large doses of acetylcholine) without modifying ganglionic transmission. HC-3 is the prototype compound for inhibiting synthesis of acetylcholine and it has been used as a tool in many published studies. There are more than 800 publications between 1980-85 which used hemicholinium as a tool. Knowledge of optimal substitution on the cationic head will be gained. Biological evaluations will include those synapses known to have considerable turnover of acetylcholine. Routine evaluation of the compounds' ability to antagonize paraoxon-induced toxicity in mice is progressing well and the test appears to be reproducible and reliable.

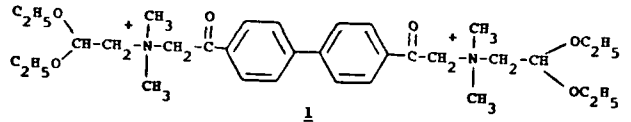
III. Approach to the problem:

This is an integrated chemistry-biology research program. The structures of newly synthesized agents are designed to provide information concerning interatomic distances between cationic moieties and spatial requirements. Our major emphasis will continue to include investigations of substitutions on the cationic head. Research outlined in this summary cites the high structural requirements for these agents. Synthesis of tertiary amines will be expanded. Biological testing will be directed toward identifying and quantifying relevant biological properties and relating these to structure. Testing of agents' ability to antagonize paraoxon-induced toxicity will proceed.

IV. Chemistry

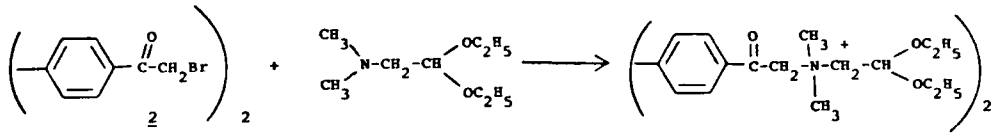
1. Synthesis of bulk quantities of compounds.

We have conducted synthesis of multi-gram quantities of "DMAE" 1.



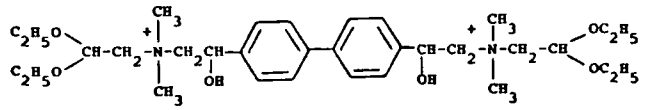
1

To this end, we have prepared sizeable amounts of 4,4'-bis-bromoacetylbiphenyl 2 and this has been permitted to react with N,N-dimethylaminoacetaldehyde,diethylacetal:



2 1

The ketonic groups of this product have been reduced to the secondary alcohol functions (structure 3), and purification of this product is underway.

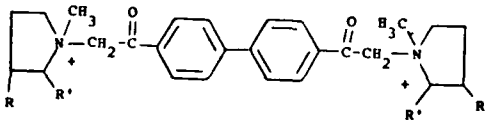


3

A multi-gram sample of the purified secondary alcohol system was submitted to Dr. Musallam for further pharmacological study.

2. Synthesis of pyrrolidine derivatives.

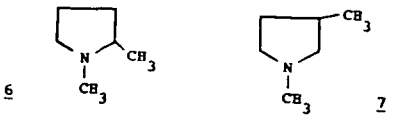
Work is continuing toward preparation of the pyrrolidine congeners 4 and 5:



4 R = H; R' = CH_3

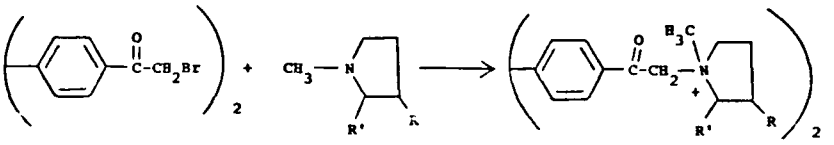
5 R = CH_3; R' = H

Prior Progress Reports described preparation of the corresponding tertiary amine systems from 4,4'-bis-bromoacetylbiphenyl and 2- and 3-methylpyrrolidines. These tertiary amines have been submitted to Profs. Long and Bhatnagar for pharmacologic studies. However, repeated and extensive attempts to quaternize these tertiary amine free bases by treatment with methyl iodide gave rise to products which were mixtures, were extremely difficult to purify, and which did not yield satisfactory elemental analyses, even though spectral (IR, NMR) data were consistent with the proposed structures. As an alternate strategy, we have prepared N-methyl-2- and 3-methylpyrrolidines 6 and 7, by Escheiler-Clarke treatment of the corresponding pyrrolidine secondary amines.



6 7

These tertiary amines have been permitted to react with 4,4'-bis-bromoacetylbiphenyl:

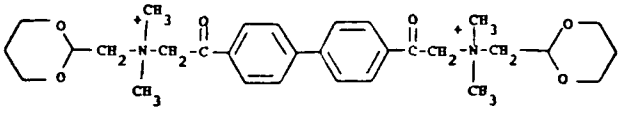


4, 5

These quaternary products seem to be much cleaner and more amenable to purification than the quaternary products obtained by quaternization with methyl iodide.

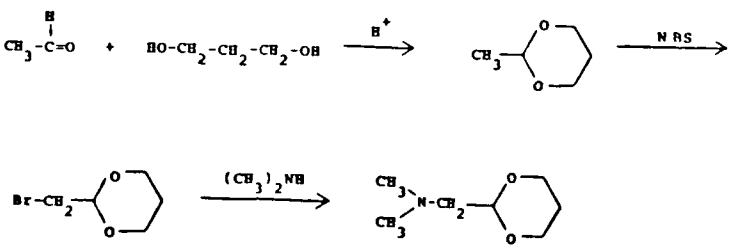
3. Synthesis of "DMAE" Derivatives.

We have initiated synthetic efforts leading to a "DMAE" derivative 8 in which the acetal moiety is a part of a 1,3-dioxane ring:



8

To this end, we have prepared 2-dimethylaminomethyl-1,3-dioxand 9 by the route shown:

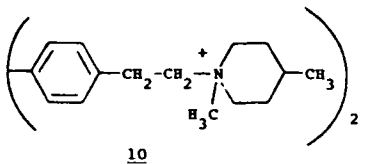


9

All steps in this sequence have been optimized and the method lends itself to scale-up reactions.

4. Synthesis of derivatives of "A-4" and "A-5".

We have initiated synthetic efforts leading to compound 10, the nonoxygenated congener of "A-4" and "A-5" (structures 11 and 12, respectively) which have been reported by our group (1-3) to have prominent HC-3-like actions in inhibiting neuromuscular transmission in rabbits and in decreasing acetylcholine content in rat caudate slices.



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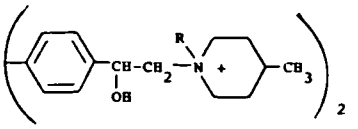
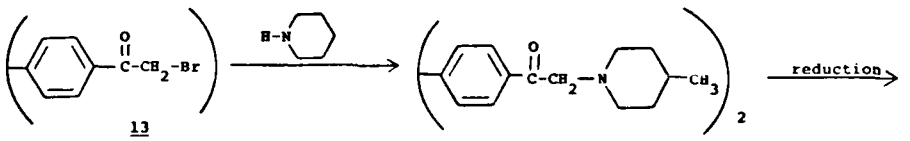


11 R = H

12 R = CH_3

We approached compound 10 as illustrated in Scheme 1.

Scheme 1. Preparation of nonoxygenated congeners of A-4 and A-5.



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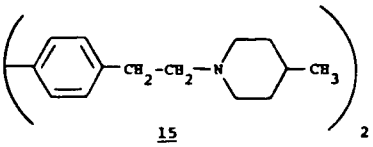
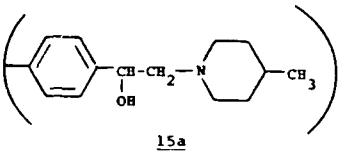
14



15

Reduction of the ketonic groups in 14 to methylene (to afford compound 15 has not been successful. It is well documented in the literature that chemical reduction methods (e.g., Clemmensen reduction under acidic conditions or Wolff-Kischner reduction under alkaline conditions) give rise to predominant reactions involving rearrangements and/or elimination of the nitrogen rings from the molecules. We concluded that catalytic hydrogenolysis would be the preferable method for converting 14 into 15. Repeated efforts to effect this simple transformation have failed. Under relatively mild conditions, the reduction stops at the benzylic alcohol stage:

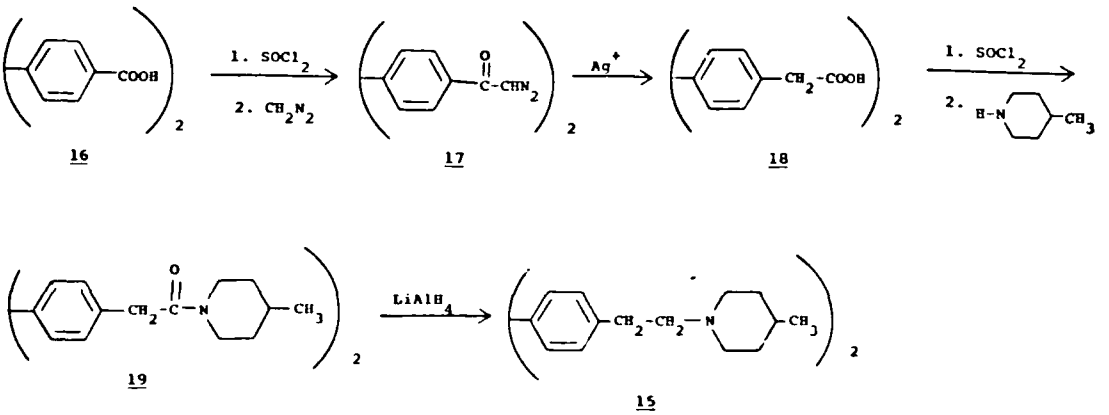


15a

More stringent reduction conditions permitted hydrogenolysis of the benzylic OH groups to methylene, but concomitantly, the aromatic rings were reduced to a bicyclohexyl system. Variations of catalyst, solvent, reaction conditions (addition of HCl or perchloric acid to promote hydrogenolysis) were not fruitful. We isolated and purified the dibenzyl alcohol 15a and subjected this material to hydrogenolysis. Again, removal of oxygen from the molecule was accompanied by reduction of the benzene rings.

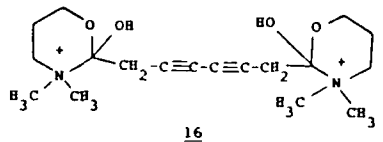
We have adopted a longer route to the target systems, shown in Scheme 2.

Scheme 2. Alternate preparation of nonoxygenated congeners.



5. Synthesis of di-acetylenic congeners of hemicholinium.

We have continued our synthetic studies, leading to the di-acetylenic congener (structure 16) of hemicholinium-3.



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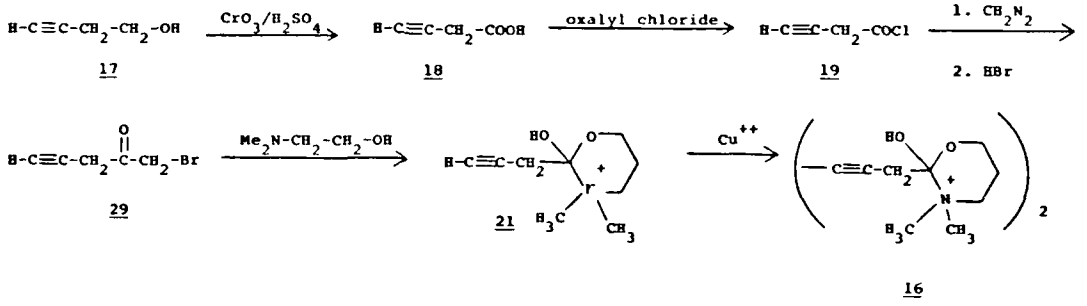
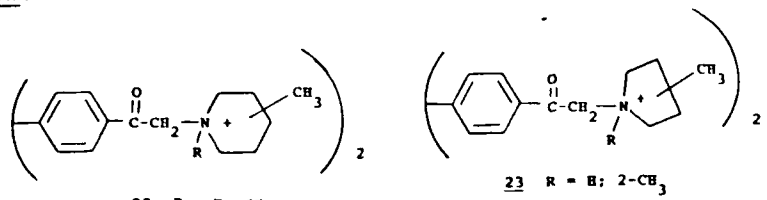
We have expanded efforts on a synthetic sequence (Scheme 1.) which we concluded shows the greatest promise:

Scheme 1. Synthetic route to di-acetylenic congener of HC-3.



It has been recognized for some time that the alpha-bromoketone 20 is a critical intermediate in the sequence. Extensive and varied efforts to prepare sufficient amounts of this material were unsuccessful. Chromatographic analysis of reaction mixtures indicated that no starting material remained, but that there was a multiplicity of products. It seems that the rate of reaction of diazomethane with the carbon-carbon triple bond is greater than the rate of reaction with the acyl chloride moiety. This route no longer seems practicable, and we have abandoned it.

Synthesis of a new series of compounds was initiated: pyrrolidine congeners (structures 23-26) of the piperidine-derived series 22:



22 R = H; CH_3

23 R = H; 2-CH_3
24 R = CH_3; 2-CH_3
25 R = H; 3-CH_3
26 R = CH_3; 3-CH_3

REFERENCES (Chemistry)

1. Tedford, C.E.; Reed, D.; Bhattacharyya, B.; Bhalla, P.; Cannon, J.G.; Long, J.P. Eur. J. Pharmacol. 1986, 128, 231.
2. Bhattacharyya, B.; Sokoll, M.D.; Cannon, J.G.; Long, J.P. Arch. Intern. Pharmacodyn. Ther. 1987, 288, 136.
3. Cannon, J.G.; Wang, Y.-F.; Sheff, K.; Tedford, C.E.; Chatterjee, T.; Bhatnagar, R.K.; Long, J.P. Submitted, Drug Design and Delivery, 1987.

V. Biology

Citations to previously reported methods.

1. Neuromuscular junction bioassay - see Annual Report No. 1, 1983.

2. Miniature end-plate potentials and voltage clamping - see Progress Report - January 1, 1986 - March 31, 1986.

3. Paraoxon-induced toxicity studies - see Progress Report - April 1, 1987 - June 30, 1987.

4. Neuroblastoma cell cultures and lipid metabolism - see Progress Report - October 1, 1987 - December 31, 1987.

5. Choline uptake studies - see Annual Report No. 2, 1984.

6. [^{3}H]Hemicholinium binding - see Progress Report - October 1, 1984 through December 31, 1984 and legends to Figures 11 and 14, this report.

7. All experimental chemicals except physostigmine and pyridostigmine were synthesized at The University of Iowa. ^{3}H-Choline chloride (80 Ci/mmole) was obtained from Amersham International, Amersham, U.K.

8. Statistics. ID_{50} and their 95% confidence limits were calculated using probit analysis (Finney, 1964). Significance in other assays was determined by Student's t-test, with significance at 0.05.

VI. Results - Biology

1. Chemicals reported in this report.

   Table I shows structures of compounds reported in the Biological Section.

2. Inhibition of neuromuscular transmission.

   Pyrrolidine derivatives (1, 2, 3, 4) were inactive in altering neuromuscular transmission at i.v. doses up to 1.1 mg/kg. All of these compounds are tertiary amines and quaternarization of the amine would be expected to enhance activity.

   Table 2 lists neuromuscular blocking activity of new compounds and those relevant to this report.

3. Miniature end-plate potentials and voltage clamping.

   Antagonism of di-isopropylflurophosphate (DFP) and paraoxon by DMAE and DMAE analogs (13, 15, 16, 19) using miniature end-plate currents (mepc's) at the neuromuscular junction has been conducted and these results are illustrated in Figs. 1 and 2 and

summarized in Tables 3 and 4. Experiments were conducted using two conventional microelectrode voltage clamp techniques in frog sartorius muscle. Paraoxon and DFP (10^{-5}M) produced a marked prolongation of time constant of decay of mepc as well as amplitude. DMAE analogs, 13 and 15, like DMAE, decreased the amplitude and accelerated the time constant of decay in a concentration dependent manner without altering the single exponential nature of mepc decay. In presence of DFP alone, mepc is bi-exponential, but after DMAE, 13 and 15, it returned to one step exponential function. These compounds induce pronounced voltage and concentration dependent nonlinearity in the current-voltage relationship. The relationship between the time constant of mepc decay and membrane potential was progressively reduced with increasing concentration. However, DMAE and 19 were found to be totally inactive in mepc amplitude or decay and 16 can decrease the amplitude of mepc without altering mepc decay.

DMAE - a bis acetal analog of HC-3 and its other analogs, 13 and 15, have been found to be highly selective for nicotinic receptor agonists including ACh (confirmed by other pharmacological studies). Hence, these types of agents appear to be selective for receptors which are activated by large amounts of agonist. Exposure to cholinesterase inhibitors produces prominent motor, behavioral and autonomic symptoms. The motor symptoms are fasciculation, fibrillation and body tremor. It was reported that fasciculation and fibrillation are due to antidromic neuronal discharge from excess junctional ACh; tremors are of central origin.

The pattern of motor symptoms that DFP produces are similar to fasciculation and myokymia produced by cholinesterase inhibitors. In this study DMAE and its analogs, 13 and 15, were studied before and after DFP. Our results indicate these anti-nicotinic compounds offer antagonism of cholinesterase inhibitors (DFP) at the motor end-plate. These effects are probably mediated by voltage dependent inhibition of ACh-activated ionic channels.

4. Antagonism of paraoxon-induced toxicity in mice.

Table 5 shows data for toxicity studies and for protective activity against paraoxon-induced lethality in mice. Physostigmine and pyridostigmine showed very high efficacy against paraoxon-induced toxicity. Dose-response relationships for the more active compounds are illustrated in Figs. 3-10.

5. Molecular mechanisms for the regulation of choline uptake and ATP-dependent conversion of striatal [^{3}H]hemicholinium-3 binding sites from high to low affinity state.

Introduction

We have shown that [^{3}H]HC-3 binding sites in rat striatal membranes exist in a high (K_D 22.3 nM) affinity state (see earlier Progress Reports and Chatterjee et al. J. Neurochem. 49: 1191-

1201, 1987). We had proposed that the two affinity states of [^{3}H]HC-3 binding sites represent the different functional states of the choline carrier system and they are convertible; the binding of HC-3 or choline to the high affinity state of the binding sites induced a change such that the binding sites are converted to a low affinity state.

The present studies were undertaken to study the underlying molecular mechanism for the interconversion of high and low affinity states of [^{3}H]HC-3 binding sites. We tested whether or not mechanisms involving phosphorylation induced different affinity states of [^{3}H]HC-3 binding to striatal membranes.

Results

The binding of [^{3}H]HC-3 to rat striatal membrane preparation yielded curvilinear Scatchard plots. The data could best be fitted to a two-site model using the LIGAND program demonstrating high (K_D 9.21 nM) and low (K_D 24.16 nM) affinity binding sites or states (Fig. 11). ATP (0.1 mM) fully converted all the binding sites to a low affinity state (Fig. 12). Increase in ATP concentration did not further change the K_D. ADP (1 mM), CTP (0.5 mM), GTP (0.5 mM) and the non-hydrolyzable analog of ATP, App(CH_2)p (1 mM), did not mimic this effect of ATP (data not shown). App(CH_2)p, however, inhibited the expression of the low affinity state of binding in the control tissue (Fig. 13). Cyclic AMP (0.5 mM), in the presence of a phosphodiesterase inhibitor, 3-isobutyl-1-methyl-xanthine (1 mM), did not affect the binding in the control tissue; when tested in combination with 0.5 mM ATP, cAMP did not further affect the binding parameters observed with ATP alone (data not shown).

The preincubation of tissue for 15 minutes at 37°C quantitatively converted the binding sites to a single high affinity state (Fig. 14). Addition of ATP (0.5 mM) again converted all the receptors from the high to a low affinity state (Fig. 15) in this preparation. Addition of App(CH_2)p did not affect the high affinity state of [^{3}H]HC-3 binding in the preincubated tissue (data not shown).

Preliminary data indicates that GTP and Gpp(NH)p (0.1 mM), but not cyclic GMP (0.5 mM), significantly reduced [^{3}H]HC-3 binding.

Discussion and Future Directions

The ATP-induced conversion of the affinity states of the [^{3}H]HC-3 binding sites suggests a definite requirement for ATP, since CTP, ADP or non-hydrolyzable analog of ATP, App(CH_2)p, cannot substitute for ATP. The inability of App(CH_2)p to substitute for ATP indicates that the hydrolysis of the terminal phosphate group of the ATP molecule is essential in inducing the conversion in the affinity states of [^{3}H]HC-3 binding sites; β-γ-methylene ATP, in contrast to ATP, prevents the expression of the low affinity

binding state in the control tissue, perhaps by an antagonism of the endogenous ATP.

The ATP-induced change in the affinity for [^{3}H]HC-3 binding does not appear to be due to an ATP-dependent proteolysis of the binding sites. Hemin (0.1 mM) and sodium vanadate (0.1 mM), the known inhibitors of ATP-dependent proteolytic activity were ineffective in preventing the ATP-induced conversion of the affinity states of [^{3}H]HC-3 binding sites (data not shown); other serine, cysteine, aspartic and metalloproteinases inhibitors, α_2-macroglobulin (2 U/mg protein) also failed to prevent this conversion.

The preincubation of the tissue at 37°C for 15 minutes quantitatively converts the [^{3}H]HC-3 binding sites to the high affinity state (Fig. 14). The inclusion of ATP in the assay medium again converts all the binding sites to the low affinity state (Fig. 15). The ATP-dependent conversion of the binding sites to a low affinity state and the preincubation-induced conversion of the binding sites to a high affinity state allows us to suggest that the [^{3}H]HC-3 binding sites represent a homogenous population of binding sites which can exist reversibly in the high and the low affinity states. The low affinity state of the binding sites are favored in the presence of ATP. The preincubation presumably depletes the endogenous ATP, thus permitting the conversion of all the binding sites to a high affinity state. Similarly, when ATP action was blocked by a non-hydrolyzable analog of ATP, App(CH_2)p, the binding sites for [^{3}H]HC-3 converted to the high affinity state.

The mechanism of this ATP-induced conversion is not known. We are conducting experiments to elucidate these mechanisms which might involve a G protein as well. Among the several possibilities, the selectivity of ATP indicates that the ATP-induced change in the [^{3}H]HC-3 binding site affinity results from a reaction involving the hydrolysis of the γ-phosphate group of the ATP molecule and perhaps leading to the phosphorylation of the binding protein or a component associated with it. Preliminary data indicate that cAMP-dependent ATP-mediated phosphorylation is not involved, although we have not yet ruled out such a possibility. Protein kinase C type reaction can also induce an ATP-dependent phosphorylation as has been reported with insulin and EGF receptors. Similar mechanisms might be operative in relation to HC-3 binding to choline carrier sites. It should be noted that we have already reported (Chatterjee et al. J. Neurochem 49: 1191-1201, 1987) that the conversion of the binding sites from a high to a low affinity state occurs only when the binding sites are occupied by HC-3 or choline and that the affinities of HC-3 and choline for the low affinity binding state correspond to their affinities for choline transport system in the rat striatal synaptosomal preparation.

Taken together, these results clearly indicate that the [^{3}H]HC-3 binding sites can reversibly exist in at least two affinity

states. The conversion of the affinity of the binding sites from the high to the low affinity state is brought about by the binding of HC-3 or choline with the high affinity state and thereby activating ATP-dependent changes (presumably phosphorylation) of the binding sites. This ATP-dependent conversion of the binding sites to the low affinity state seems to be important in the activation of the choline transport process. The functional implication of the low affinity [^{3}H]HC-3 binding state with choline transport is supported from our findings that the developmental expression of the low affinity form of the striatal [^{3}H]HC-3 binding sites paralleled the appearance of sodium dependent high affinity choline transport system (Chatterjee and Bhatnagar, Fed. Proc. 46: 856, 1987). The results of preliminary studies suggest that ATP-sensitivity of [^{3}H]HC-3 binding is not limited to striatal tissue and other areas of the brain (e.g., hippocampus) shown similar sensitivity. Thus, ATP-dependent conversion of the HC-3 (choline) binding sites to the low affinity state might represent a general phenomenon of the choline carrier system and might regulate the functional activity of this system. Because the low affinity state of the [^{3}H]HC-3 binding sites is also sensitive to the effect of GTP and Gpp(NH)p (preliminary data), we also suggest that the functional form of the choline carrier system may also be linked with a G protein.

6. Evaluation of chemicals for inhibition of choline transport.

   a. Compound 12 is the most potent inhibitor of choline transport in the rat striatal synaptosomal preparation. Studies are underway to study the characteristics and mechanisms for this inhibition. Comparative studies will be done with 6 and 12. When radiolabeled 6 and 12 become available, competition and other binding studies will be done.

   b. Testing of most chemical entities has been completed. A comparison of the hemiacetal and piperidine derivatives is shown in Table 6. Note the lack of significant activity for compound 25 which has a favorable therapeutic index for antagonism of paraoxon-induced toxicity in mice.

7. Inhibition of choline transport in neuroblastoma cells.

   The objectives of these studies are to characterize the choline transport system in this preparation and to evaluate the influence of inhibitors of choline transport on lipid metabolism.

   a. Sodium dependency for inhibition by 5, 6 and HC-3.

      The data in Table 7 indicate that inhibition by 6 and HC-3 is sodium dependent. Compound 5 is most active in the presence of sodium, but significant inhibition is seen in the absence of sodium. Possible significance of this finding is presently being evaluated.

b. Kinetics for inhibition by 5, 6 and HC-3.

Table 8 shows that with 6 the K_m^{app} value is increased about 3.5 times while the v_{max} is not different from control. Compound 5 increased K_m^{app} approximately 6-fold and v_{max} is increased approximately 3-fold.

c. Intracellular choline concentrations of choline following 1, 2 and 3-day incubations.

With 5 there was about 30% inhibition on day 1, but the cellular choline concentrations returned to control by day 2 and appeared to increase above control levels by day 3. No significant activity was found for 6 or HC-3. Graphic representation of the above data is shown in Fig. 16.

d. Incorporation of choline into lipid.

Table 9 shows that compounds 5 and 6 are capable of decreasing the amount of choline incorporated into phospholipid of synaptosomes.

In summary, compound 5 shows a pattern for inhibition that appears to be different from 6 or HC-3. Compound 5 would be expected to be very lipid-soluble and perhaps the compound involves sites of action differing from quaternary derivatives.

8. Functional receptor studies.

a. Table 10 shows the ability of DMAE and analogs to antagonize the nicotinic-receptor stimulating action of acetylcholine in isolated rectus abdominis muscle of frogs. These compounds are active antagonists in this rather insensitive preparation. Compounds 25 and 26 will be evaluated using this preparation.

b. Table 11 shows data obtained for inhibition of nicotinic receptors using isolated guinea-pig atria. DMAE and analogs are very effective antagonists of nicotine in this preparation. This is a very sensitive assay for evaluating anti-nicotinic agents.

9. Isomers of compounds 5 and 6.

Studies have continued using the isomeric forms of 5 and 6 in neuroblastoma cells. Results are shown in Table 12. Also during the past year additional studies were completed using the rabbit neuromuscular junction. Summation of results from these studies is shown in Table 13.

In the above studies and previously reported studies, isomeric forms of compounds 5 and 6 have been shown to have similar biological activity in most preparations. This indicates that the hydroxyl group in the B-position of the side chain is not

involved in interaction with receptors to induce inhibition of the choline transport system. However, there are features about compound 5 that remain to be clarified. These would include (a) enhancing choline transport in synaptosomes in very low concentrations, 5 nM, and 5 is a more active inhibitor in synaptosomes than would be expected (IC50 30 nM) (Table 14). Also kinetic studies using 5 with neuroblastoma cells may indicate mixed facilitation and inhibition of choline transport, see Table 8.

VII. Conclusions.

1. New concepts for cholinergic mechanisms.

a. Studies evaluating mechanisms for inhibition of synaptosomal choline uptake by different hemicholinium-3 analogs reveal existence of two different classes of choline uptake inhibitors. One class inhibits choline uptake competitively and the other noncompetitively. The prototype of the former class is hemicholinium-3, while for the latter class prototype agents appear to be 6 or 12.

The effect of compound 6 is selective for the choline carrier system, since other uptake systems tested are not affected. Furthermore, the effect of 6 is at best only partially reversible by choline and is high affinity. These characteristics lead us to believe that 6's action is mediated through a site distinct from those recognized by choline. The significance of this is as yet unknown and its role in physiologic regulation of choline uptake remains to be established. Unfortunately, lack of availability of radiolabeled 6 has hampered progress in this area.

Similar to 6, the effect of hemicholinium-3 on choline uptake system is selective, of high affinity and is reversible. However, unlike 6, hemicholinium-3 inhibition of choline uptake is competitive. The availability of radiolabeled hemicholinium-3 made it possible to understand the molecular events involved in choline binding to its carrier system and to its uptake inside neurons. [^{3}H]Hemicholinium-3 bound to choline carrier sites with high and low affinity. The binding of choline or hemicholinium-3 with the high affinity form of the carrier system induced an ATP-dependent change to the low affinity state. This ATP-dependent conversion of the affinity states of the choline carrier system is brought about, presumably, by the transfer of the gamma-phosphate group of ATP to the [^{3}H]HC-3 binding component or a component associated with it. This ATP-dependent conversion of the carrier system involves a membrane bound component that can easily be washed off from the membrane by 150 mM NaCl.

Preliminary studies also indicate that GTP could effect the binding of hemicholinium-3 with the choline carrier system. In the presence of GTP no membrane binding of hemicholinium-

3 was detectable. The mechanism of GTP-induced alteration of [^{3}H]HC-3 binding is currently under investigation.

On the basis of these studies, we postulate that the choline carrier system exists in two forms, a "nonfunctional" and a "functional" form; occupancy of the nonfunctional choline carrier sites with choline induces ATP-dependent phosphorylation of the sites and produces a functional choline carrier system.

2. Status of studies involving protective agents for paraoxon-induced toxicity in mice.

   a. Compounds 13, 15 and 16 are being prepared in bulk synthesis for your protective studies.

   b. The recent discovery that compounds 25 and 26 have a protective therapeutic index very similar to physostigmine and pyridostigmine must be regarded as encouraging leads for this research. Compound 25 has also been shown to be a potent antagonist in electrophysiological studies against paraoxon, (Fig. 17). At this time it would be premature to postulate mechanisms of action or structure activity relationships.

   c. Physostigmine and pyridostigmine are dramatic antagonists of paraoxon-induced toxicity in mice. In the presence of atropine, we doubt that one could demonstrate any pharmacological responses with doses of physostigmine or pyridostigmine which show significant protection (i.e., 10 µg/kg IM). It may be that one could not demonstrate inhibition of cholinesterase with this dose; these agents as inhibitors of cholinesterase are not very potent. Molecular interaction between these chemicals and paraoxon does not seem to be a reasonable mechanism because there are more than 300 molecules of paraoxon present for each molecule of physostigmine. The mechanism may involve alkylation of the "esteratic site" of cholinesterase resulting in protection from phosphorylation by the O-P. However, not a lot is known about the pharmacology of physostigmine and pyridostigmine other than inhibition of cholinesterase. Could these agents alter the synthesis or release of ACh by a neuron? Could there be interactions between membrane-bound acetylcholinesterase, these chemicals and choline? Perhaps data from future experiments involving our potent inhibitors of cholinesterase, which would not alkylate a receptor, will help form a more valid hypothesis.

VIII. Recommendations.

These chemicals have been and are powerful tools to discover and study cholinergic mechanisms. Fortunately, these series of compounds modify cholinergic transmission by several distinct mechanisms so the issues of science which are raised may appear broad.

Analogs of DMAE (anti-nicotinic) are effective against paraoxon-induced toxicity in mice. At least we presume that the protective action is related to anti-nicotinic activity. The molecular mechanism of these agents is totally unknown. Their protective actions do not correlate with inhibition of choline transport.

Studies will continue to determine the molecular mechanisms involved in choline transport. Experiments in this report are cited which indicate that molecular mechanisms for choline transport may not be greatly different from those which have been postulated for various receptor mechanisms. Certainly, this is an area of limited knowledge and with the availability of new radioligands, progress should be forthcoming.

Agents will continue to be evaluated for their protective action against paraoxon-induced toxicity. As discussed above, it appears that we have new leads introduced into this research by compounds 25 and 26. This area should receive extensive chemical and biological efforts. Studies must be conducted to learn more about the pharmacological properties of this class of chemicals.

IX. Publications resulting from this research are shown below.

1. C.E. Tedford, M.J. Schott, J.R. Flynn, J.G. Cannon and J.P. Long, A-4, A bis tertiary amine derivative of hemicholinium-3 produce in vivo reduction of acetylcholine in rat brain regions, J. Pharmacol. Exp. Ther., 240, 476-485 (1987).

2. J.G. Cannon, T.M.-L. Lee, A.M. Nyanda, B. Bhattacharyya and J.P. Long, Structure-activity relationship studies in the hemicholinium ("HC-3") series, Drug Design and Delivery, 1, 209-218 (1987).

3. T.K. Chatterjee, J.G. Cannon and R.K. Bhatnagar, Characteristics of [^{3}H]hemicholinium binding to rat striatal membranes: evidence for negative cooperative site-site interaction. J. Neurochem., 49, 1191-1201 (1987).

4. B. Bhattacharyya, M.D. Sokoll, J.G. Cannon and J.P. Long, Pharmacological evaluation and structure activity relationships of a series of hemicholinium (HC-3) analogs, Arch. Int. Pharmacodyn. 288, 136-146 (1987).

5. B. Bhattacharyya, M.D. Sokoll, H.G. Cannon and J.P. Long, Neuromuscular blocking action of two hemicholinium-3 analogs, Eur. J. Pharmacol., 146, 155-165 (1988).

6. J.G. Cannon, Y.-F. Wang, K. Sheff, C.E. Tedford, T. Chatterjee, R.K. Bhatnagar and J.P. Long, Optical isomers of some piperidine-derived hemicholinium congeners containing secondary alcohol groups: preparation and biological activities, Drug Design and Delivery, in press.

7. J.G. Cannon, T.M.-L. Lee, Y.-A. Chang, A.M. Nyanda, B. Bhattacharyya, J.R. Flynn, T. Chatterjee, R.K. Bhatnagar and J.P. Long, Structure-activity relationship studies of hemicholinium ("HC-3") congeners, Pharmaceutical Research, in press.

8. T.K. Chatterjee, J.P. Long, J.G. Cannon and R.K. Bhatnagar, Methylpiperidine analog of hemicholinium-3: a selective high affinity noncompetitive inhibitor of sodium-dependent choline uptake system, Eur. J. Pharmacol., in press.

9. C.E. Tedford, J.R. Flynn, R.K. Bhatnagar, J.G. Cannon and J.P. Long, Alteration in acetylcholine metabolism in rat striatal slices by a 4-methyl piperidine analog of hemicholinium-3, J. Pharmacol. Exp. Therap., in press.

10. K. Sheff, C.E. Tedford, J.R. Flynn, B. Bhattacharyya, M.A. Yorek and J.P. Long, Stereoisomeric 4-methyl piperidine analogs of hemicholinium-3, J. Pharmacol., Exp. Therap., submitted.

X. Figure Legends

Fig. 1. Antagonism of irreversible cholinesterase inhibitor DFP in frog rectus abdominis muscle by compound 13 (TL-402).

Fig. 2. Antagonism of paraoxon in from rectus abdominis muscle by compound 15 (NAM-242).

Figs. 3-10 - Antagonism of paraoxon-induced lethality in mice.

3. Physostigmine.

4. Pyridostigmine.

5. Compound 25.

6. Compound 13.

7. Compound 15.

8. Compound 16.

9. Compound DMAE.

10. Compound 19.

Figure 11: Scatchard plot of [^{3}H]HC-3 binding with rat striatal membrane preparations. The rat striatal region was homogenized in 50 volumes of ice-cold 10 mM sodium potassium phosphate buffer (pH 7.4 at 25°C) using a Polytron (setting 7, 20 s). The homogenate was centrifuged for 10 minutes at 48,000 x g, and the resulting pellet was washed twice in the homogenization buffer by rehomogenizing and centrifuging. The pellet was finally suspended in 10 mM sodium phosphate buffer containing 150 mM NaCl to yield a final concentration of 50 mg of original wet weight of tissue/ml (approximately 5 mg protein/ml). The standard [^{3}H]HC-3 binding assay mixture contained 50 µl of membrane solution, 20 µl of [^{3}H]HC-3 and 10 mM of sodium phosphate buffer containing 150 mM NaCl to give a final volume of 100 µl. Nonspecific binding was defined as that obtained in the presence of 10 µM unlabeled HC-3. [^{3}H]HC-3 binding was done by incubating the membranes with varying concentrations of [^{3}H]HC-3 at 35°C for 40 minutes.

Data are from a representative experiment repeated at least four times. Linearity of the Scatchard plot was evaluated by applying runs test (Zer, J.H., Biostatistical Analysis, Prentice Hall, Englewood Cliffs, NJ, 1984). The plots deviating significantly from linearity (this figure) were analyzed by using the graphic analysis of DeMeyts, P. and J. Roth (Biochem. Biophys. Res. Commun. 66: 1118-1126, 1975). The data yielding linear Scatchard plots were analyzed by using the LIGAND program (Munson, P. and D. Rudbard, Anal. Biochem. 107: 220-239, 1980) with the modification for adaptation to microcomputers (McPharson, G.A., 1985; A Collection of Radioligand Binding Analysis Programs; Elsevier Science Publishers, BV, Amsterdam). The subscripts H and L indicate high- and low-affinity states, respectively.

Figure 12: Scatchard plot of [^{3}H]HC-3 binding with rat striatal membrane preparations in the presence of 0.1 mM ATP. [^{3}H]HC-3 binding and the analysis of data were done as described in the legend for Figure 11.

Figure 13: Scatchard plot of [^{3}H]HC-3 binding with rat striatal membrane preparations in the presence of the nonhydrolyzable analog of ATP, App(CH_2)p. [^{3}H]HC-3 binding and the analysis of data were done as described in the ligand for Figure 11.

Figure 14: Scatchard plot of [^{3}H]HC-3 binding with rat striatal membrane preparations from tissue that was preincubated at 37°C for 15 minutes. The striatal tissue was homogenized in 50 volumes of ice-cold 10 mM sodium potassium phosphate buffer (pH 7.4 at 25°C) using a Polytron. The homogenates were centrifuged and the resulting pellet was suspended in buffer and preincubated at 37°C for 15 minutes before washings and preparation of membranes as described in the legend for Figure 11. [^{3}H]HC-3 binding and the analysis of data were done as described in the legend for Figure 11.

Figure 15: Scatchard plot of [^{3}H]HC-3 binding, in the presence of ATP, with rat striatal membrane preparations from tissue that was preincubated at 37°C for 15 minutes. Membranes were prepared as described in the legend for Figure 14 and [^{3}H]HC-3 binding and analysis of data were done as described in the legend to Figure 11.

Figure 16: Intracellular concentrations of choline following 1-, 2- and 3-day incubations of neuroblastoma cells with compound 5, compound 6 and HC-3. Compound 5 showed a 30% decrease on day 1 and choline concentration returned to control on day 2 and was increased by day 3. Compound 6 and HC-3 had no significant effect.

Figure 17: The antagonistic action of compound 25 (CA-6) against paraoxon-induced end-plate current changes in frog sartorius muscle.

FIGURE 1

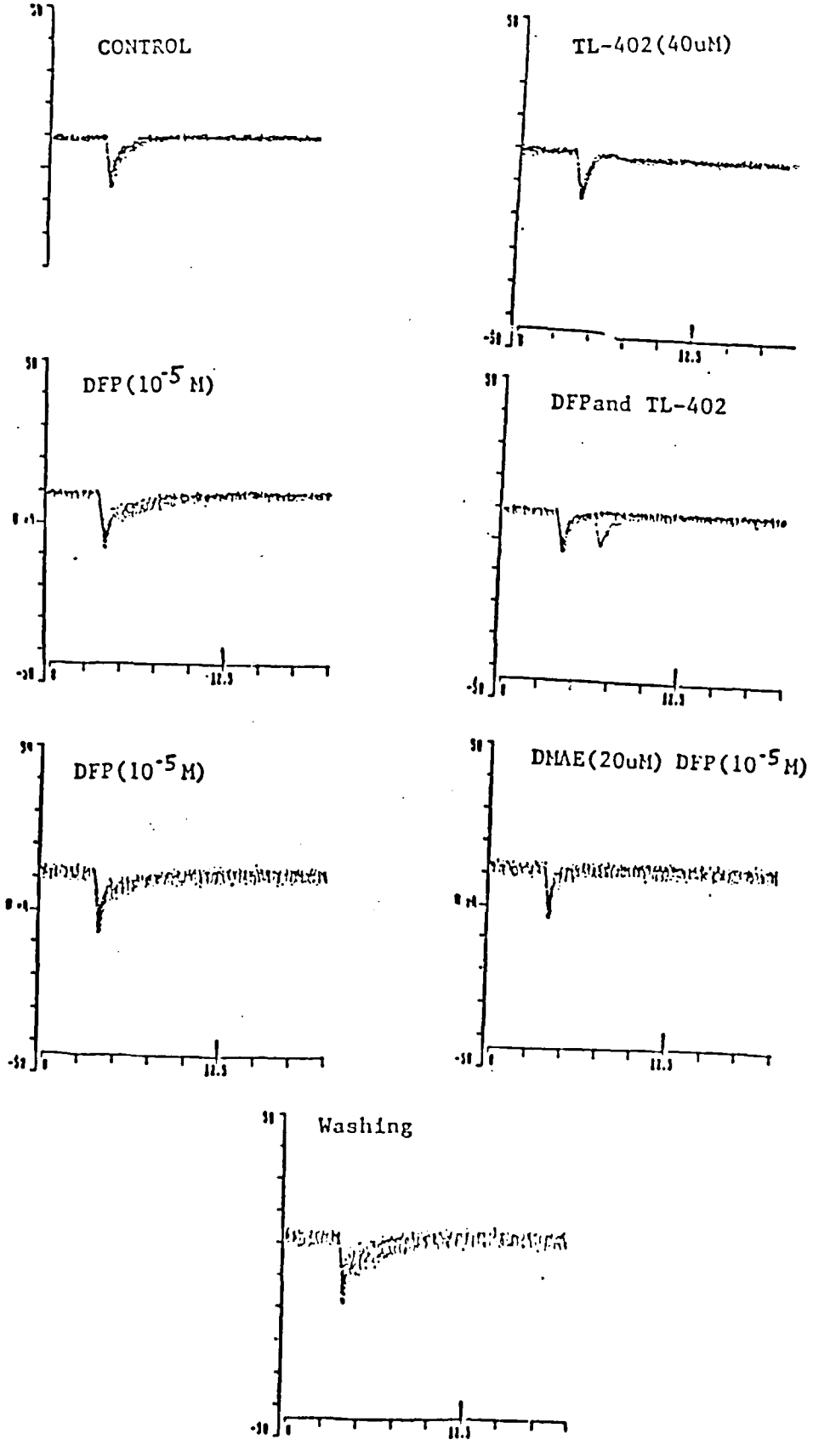

CONTROL
TL-402(40uM)
DFP(10^{-5} M)
DFPand TL-402
DFP(10^{-5} M)
DMAE(20uM) DFP(10^{-5} M)
Washing

FIGURE 2

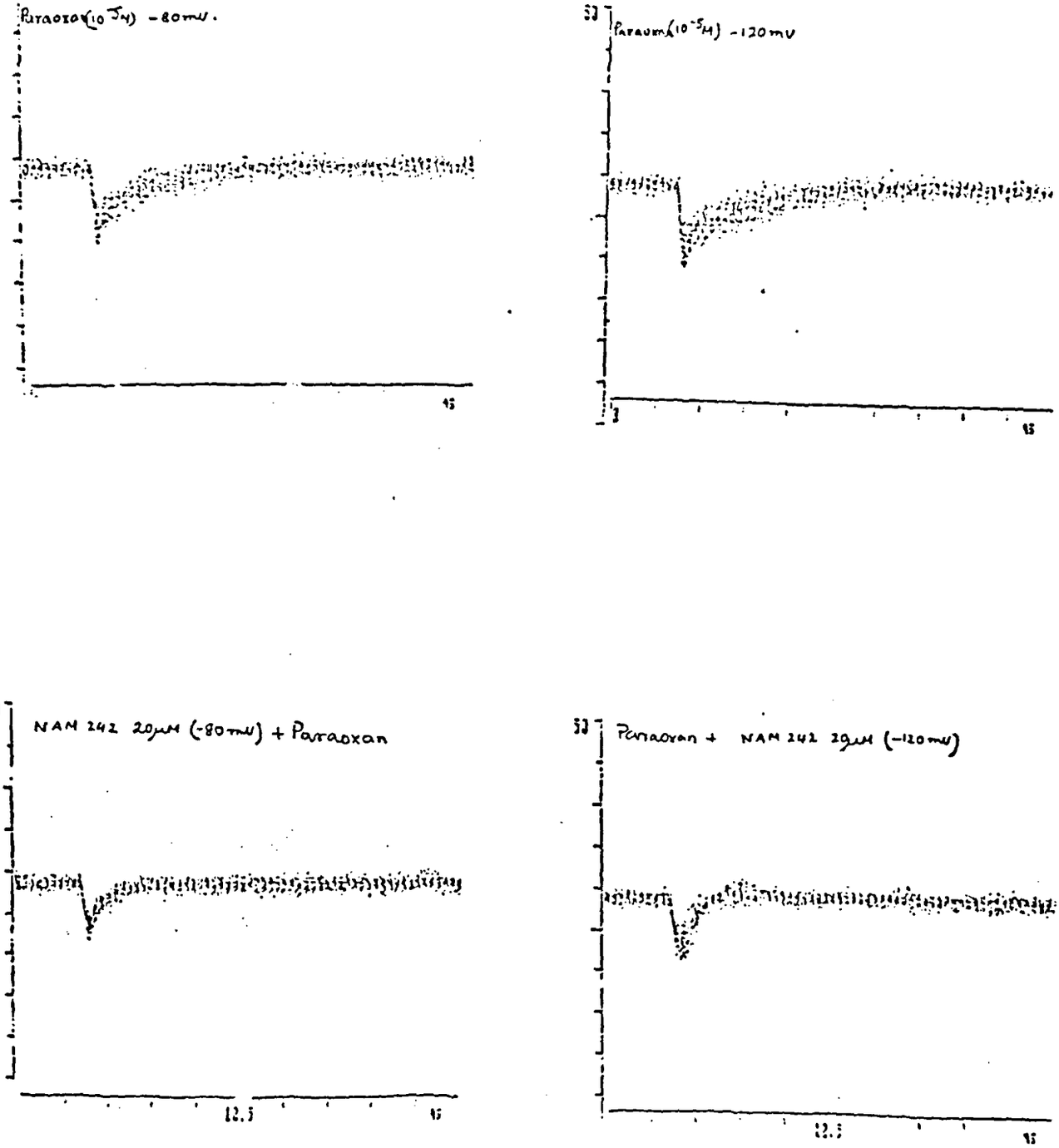

Paraoxon (10^{-5}M) -80mV.
Paraoxon (10^{-5}M) -120mV
NAM 242 20μM (-80mV) + Paraoxan
Paraoxan + NAM 242 20μM (-120mV)

FIGURE 3

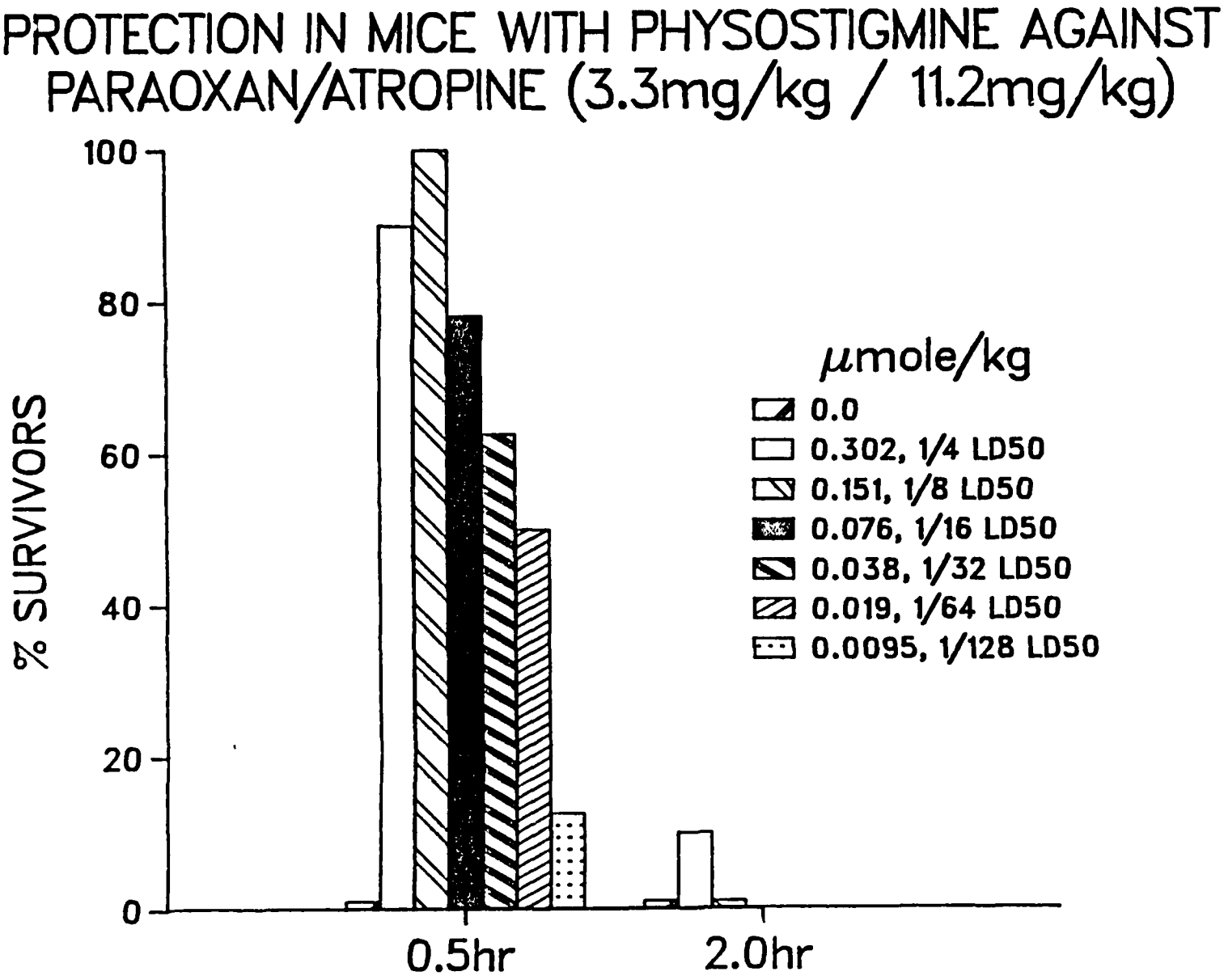

PROTECTION IN MICE WITH PHYSOSTIGMINE AGAINST PARAOXAN/ATROPINE (3.3mg/kg / 11.2mg/kg)
% SURVIVORS
100
80
60
40
20
0
0.5hr
2.0hr
μmole/kg
0.0
0.302, 1/4 LD50
0.151, 1/8 LD50
0.076, 1/16 LD50
0.038, 1/32 LD50
0.019, 1/64 LD50
0.0095, 1/128 LD50

FIGURE 4

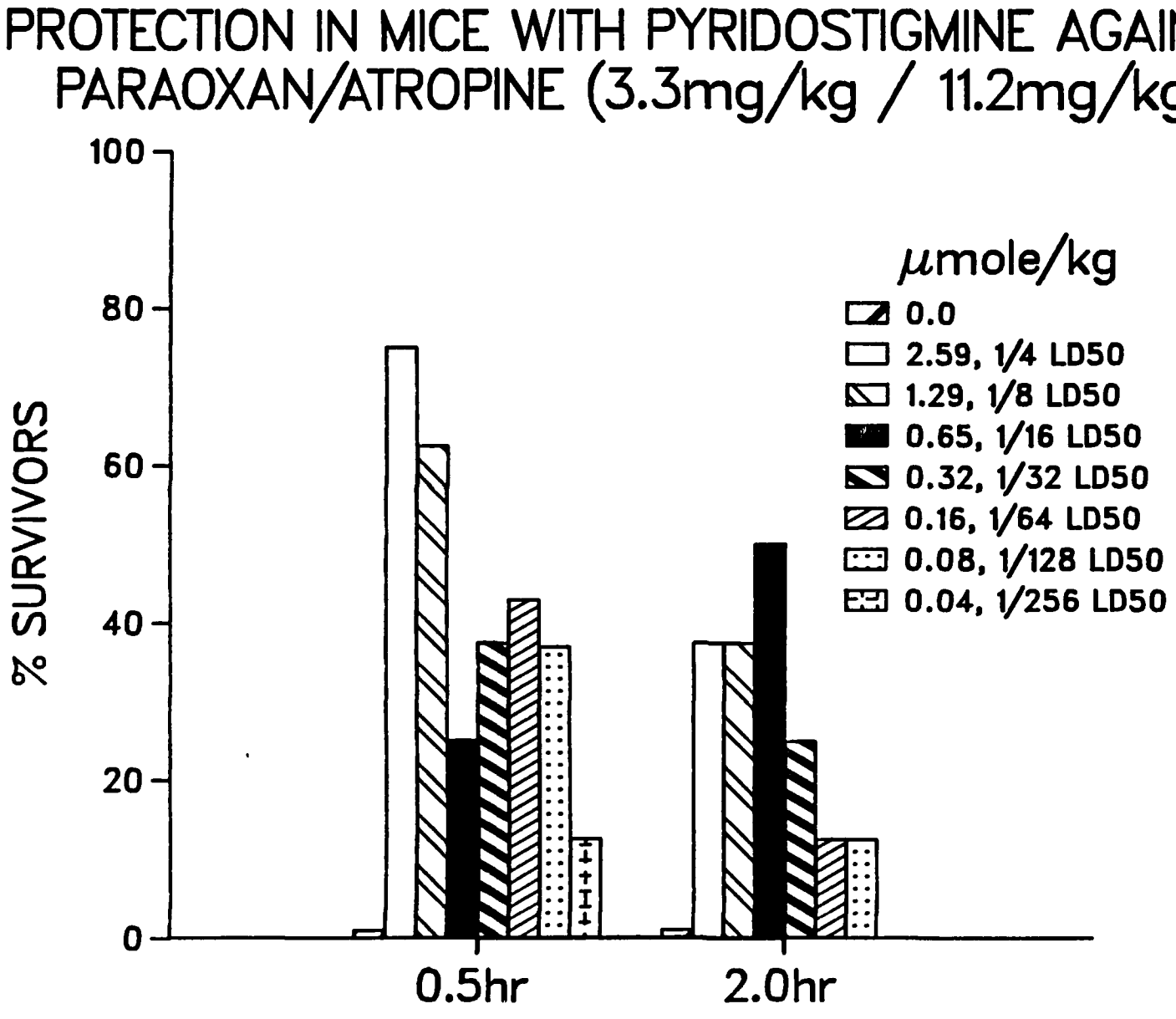

PROTECTION IN MICE WITH PYRIDOSTIGMINE AGAINST PARAOXAN/ATROPINE (3.3mg/kg / 11.2mg/kg)
% SURVIVORS
100
80
60
40
20
0
0.5hr
2.0hr
μmole/kg
0.0
2.59, 1/4 LD50
1.29, 1/8 LD50
0.65, 1/16 LD50
0.32, 1/32 LD50
0.16, 1/64 LD50
0.08, 1/128 LD50
0.04, 1/256 LD50

FIGURE 5

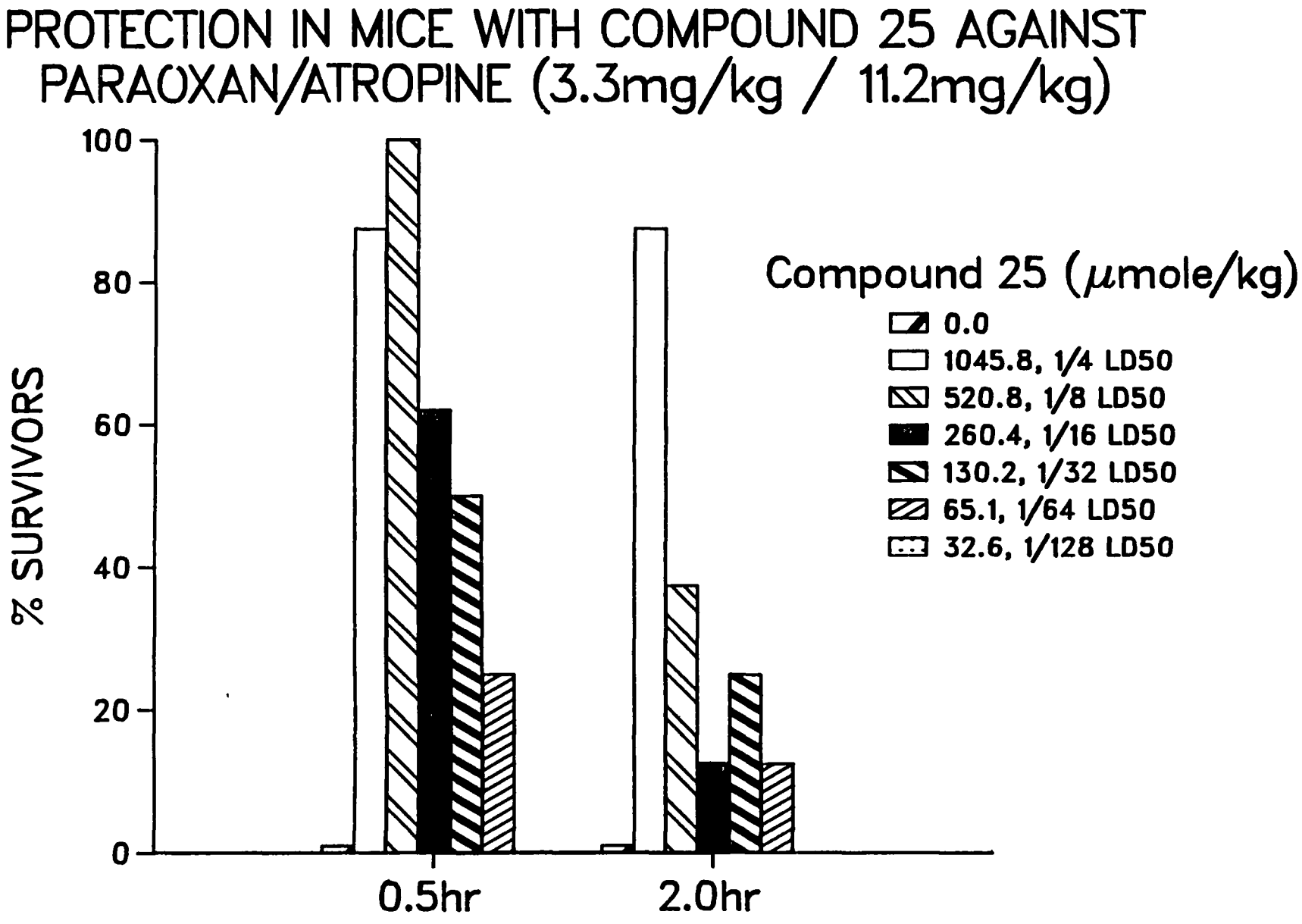

PROTECTION IN MICE WITH COMPOUND 25 AGAINST PARAOXAN/ATROPINE (3.3mg/kg / 11.2mg/kg)
% SURVIVORS
100
80
60
40
20
0
0.5hr
2.0hr
Compound 25 (μmole/kg)
0.0
1045.8, 1/4 LD50
520.8, 1/8 LD50
260.4, 1/16 LD50
130.2, 1/32 LD50
65.1, 1/64 LD50
32.6, 1/128 LD50

FIGURE 6

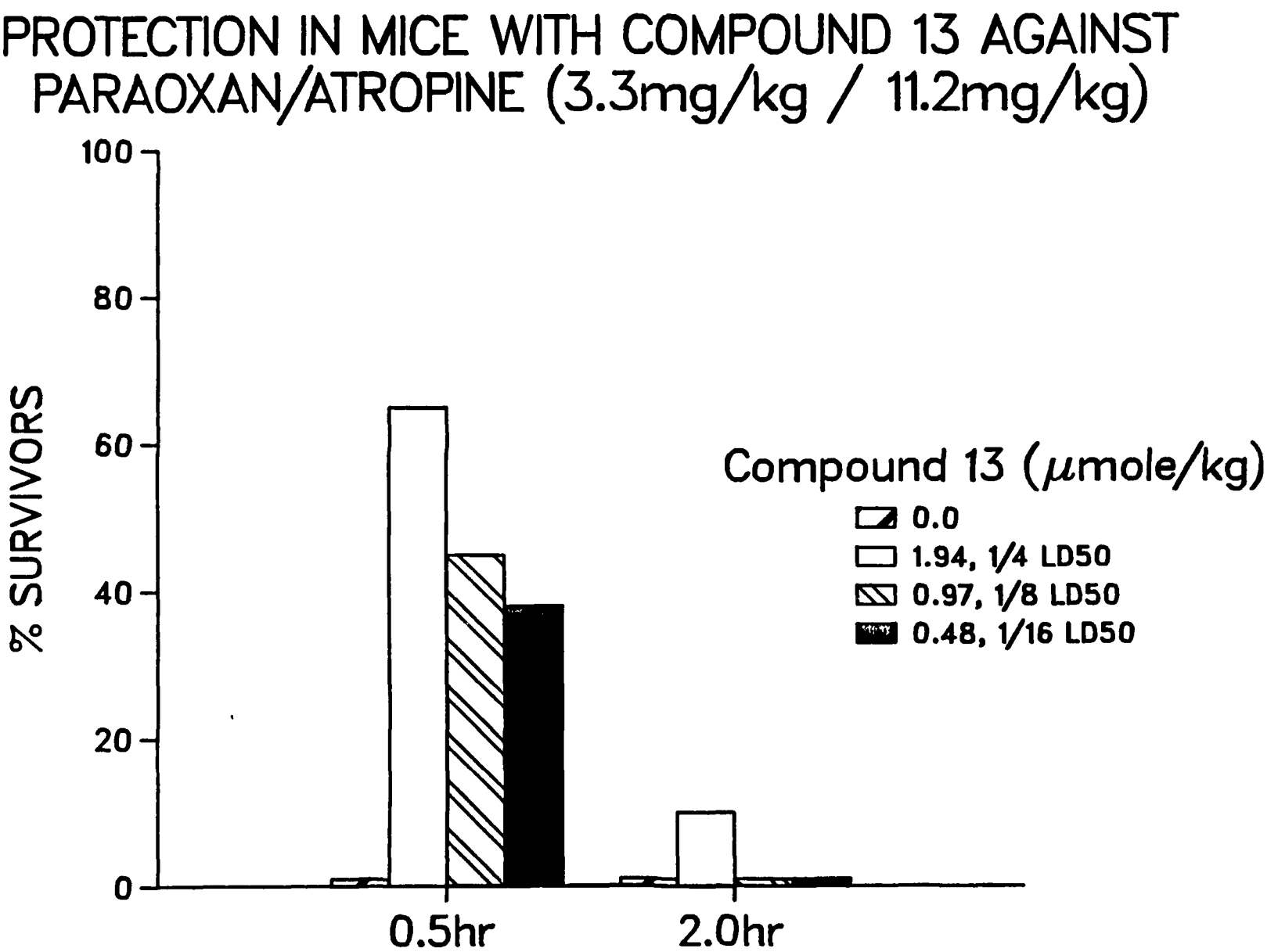

PROTECTION IN MICE WITH COMPOUND 13 AGAINST PARAOXAN/ATROPINE (3.3mg/kg / 11.2mg/kg)
% SURVIVORS
100
80
60
40
20
0
0.5hr
2.0hr
Compound 13 (μmole/kg)
0.0
1.94, 1/4 LD50
0.97, 1/8 LD50
0.48, 1/16 LD50

FIGURE 7

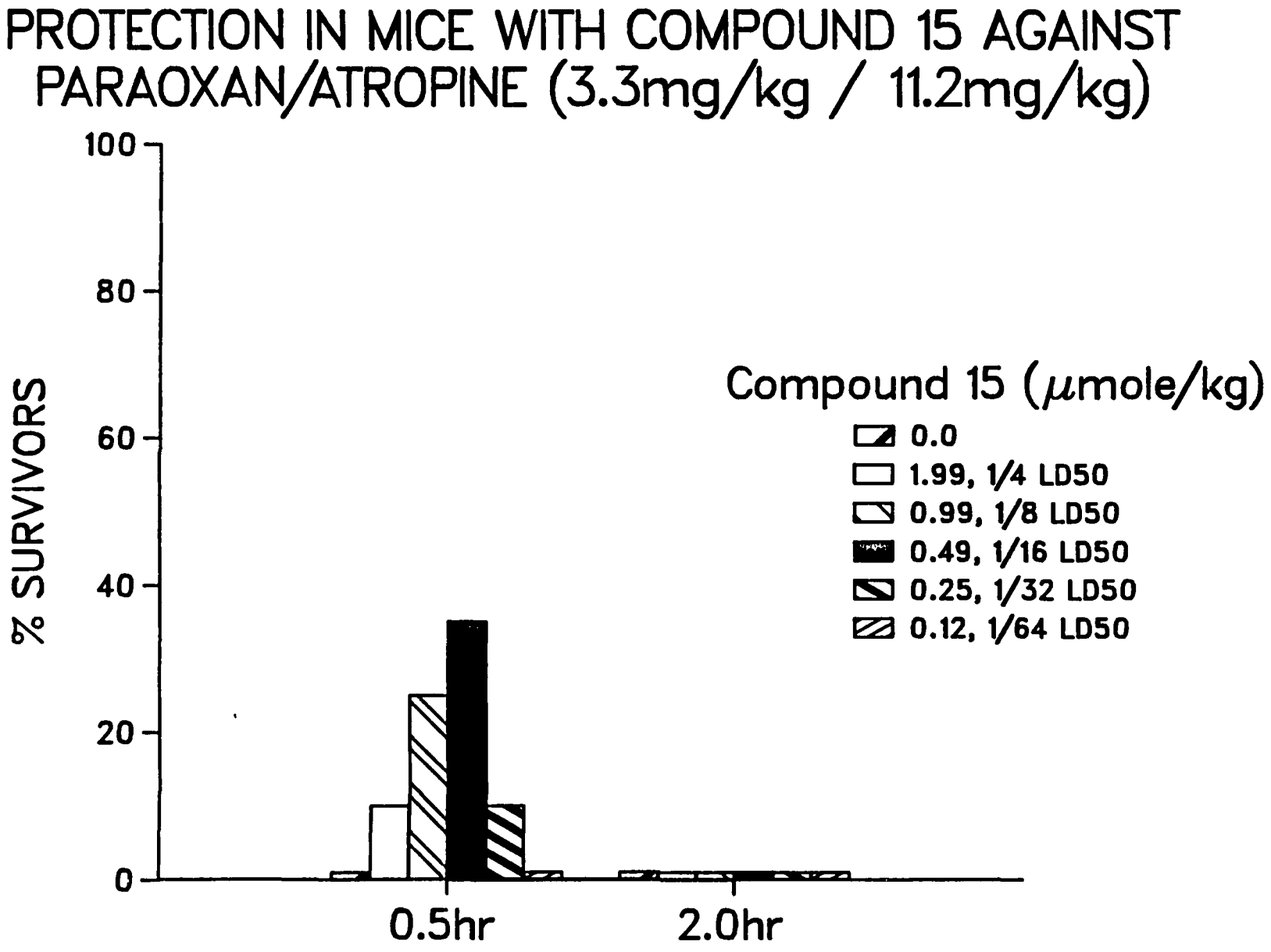

PROTECTION IN MICE WITH COMPOUND 15 AGAINST PARAOXAN/ATROPINE (3.3mg/kg / 11.2mg/kg)
% SURVIVORS
100
80
60
40
20
0
0.5hr
2.0hr
Compound 15 (μmole/kg)
0.0
1.99, 1/4 LD50
0.99, 1/8 LD50
0.49, 1/16 LD50
0.25, 1/32 LD50
0.12, 1/64 LD50

FIGURE 8

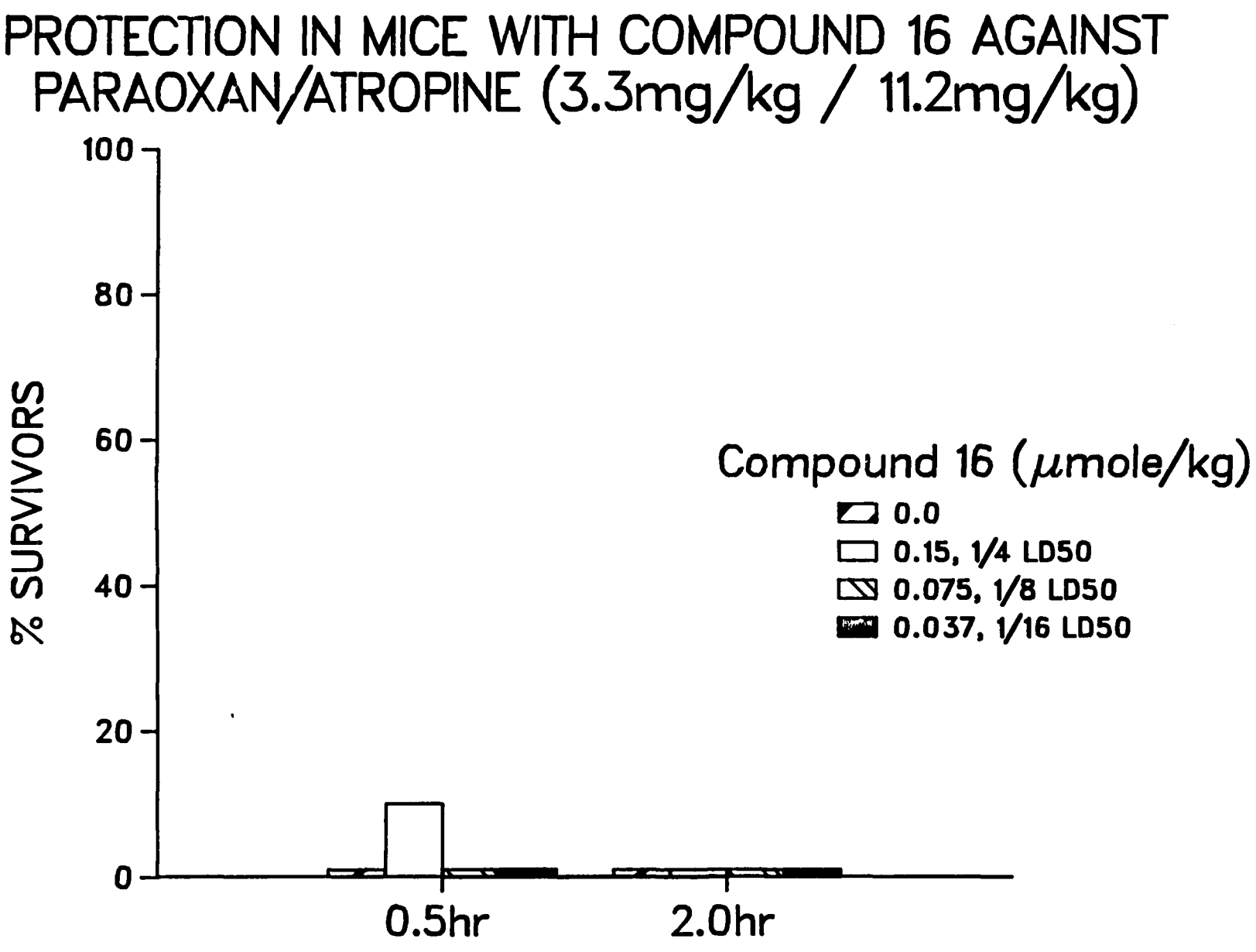

PROTECTION IN MICE WITH COMPOUND 16 AGAINST PARAOXAN/ATROPINE (3.3mg/kg / 11.2mg/kg)
% SURVIVORS
100
80
60
40
20
0
0.5hr
2.0hr
Compound 16 (μmole/kg)
0.0
0.15, 1/4 LD50
0.075, 1/8 LD50
0.037, 1/16 LD50

FIGURE 9

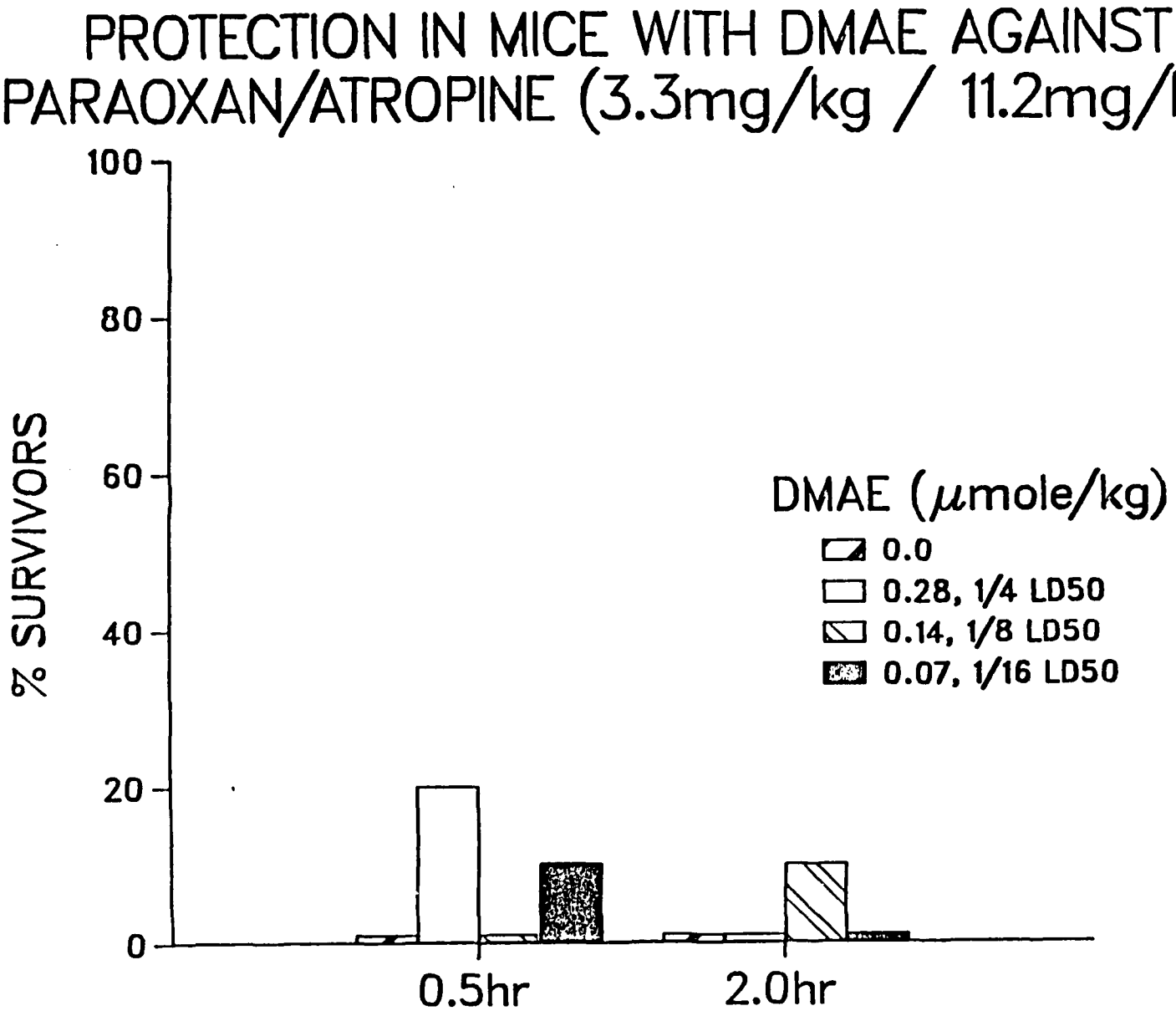

PROTECTION IN MICE WITH DMAE AGAINST PARAOXAN/ATROPINE (3.3mg/kg / 11.2mg/kg)
% SURVIVORS
100
80
60
40
20
0
0.5hr
2.0hr
DMAE (μmole/kg)
0.0
0.28, 1/4 LD50
0.14, 1/8 LD50
0.07, 1/16 LD50

FIGURE 10

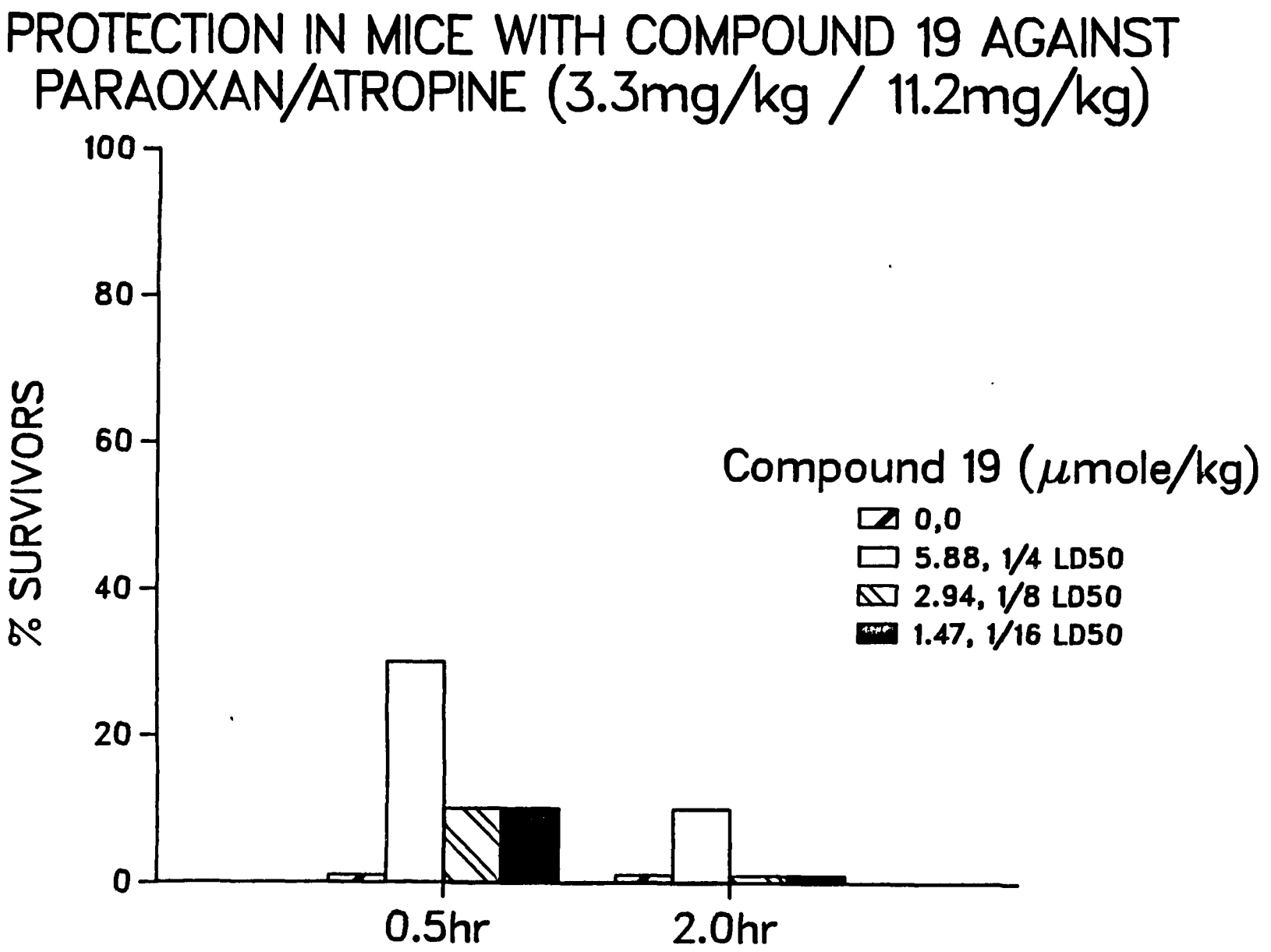

PROTECTION IN MICE WITH COMPOUND 19 AGAINST PARAOXAN/ATROPINE (3.3mg/kg / 11.2mg/kg)
% SURVIVORS
100
80
60
40
20
0
0.5hr
2.0hr
Compound 19 (μmole/kg)
0,0
5.88, 1/4 LD50
2.94, 1/8 LD50
1.47, 1/16 LD50

FIGURE 11

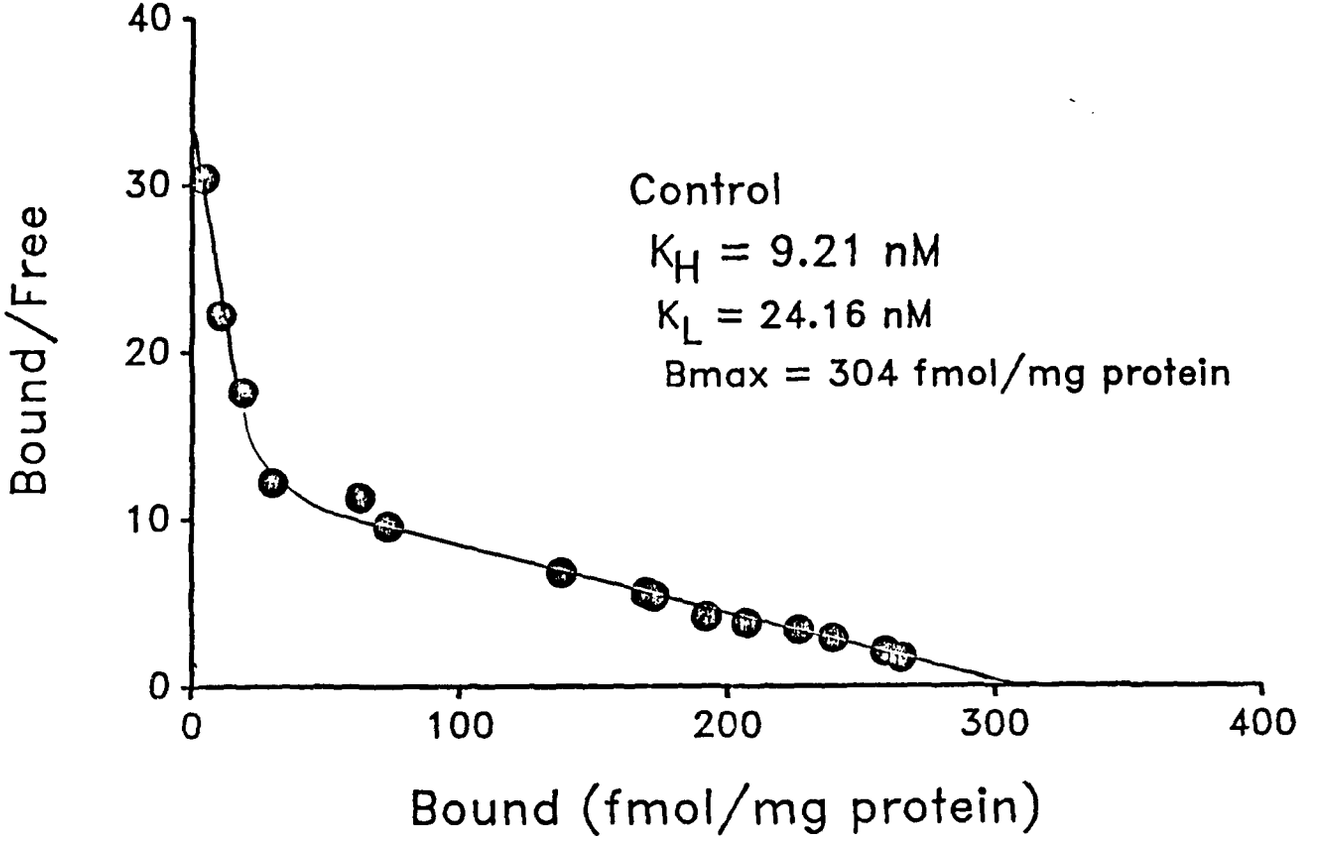

Control
K_H = 9.21 nM
K_L = 24.16 nM
Bmax = 304 fmol/mg protein
Bound/Free
Bound (fmol/mg protein)
0
10
20
30
40
0
100
200
300
400

FIGURE 12

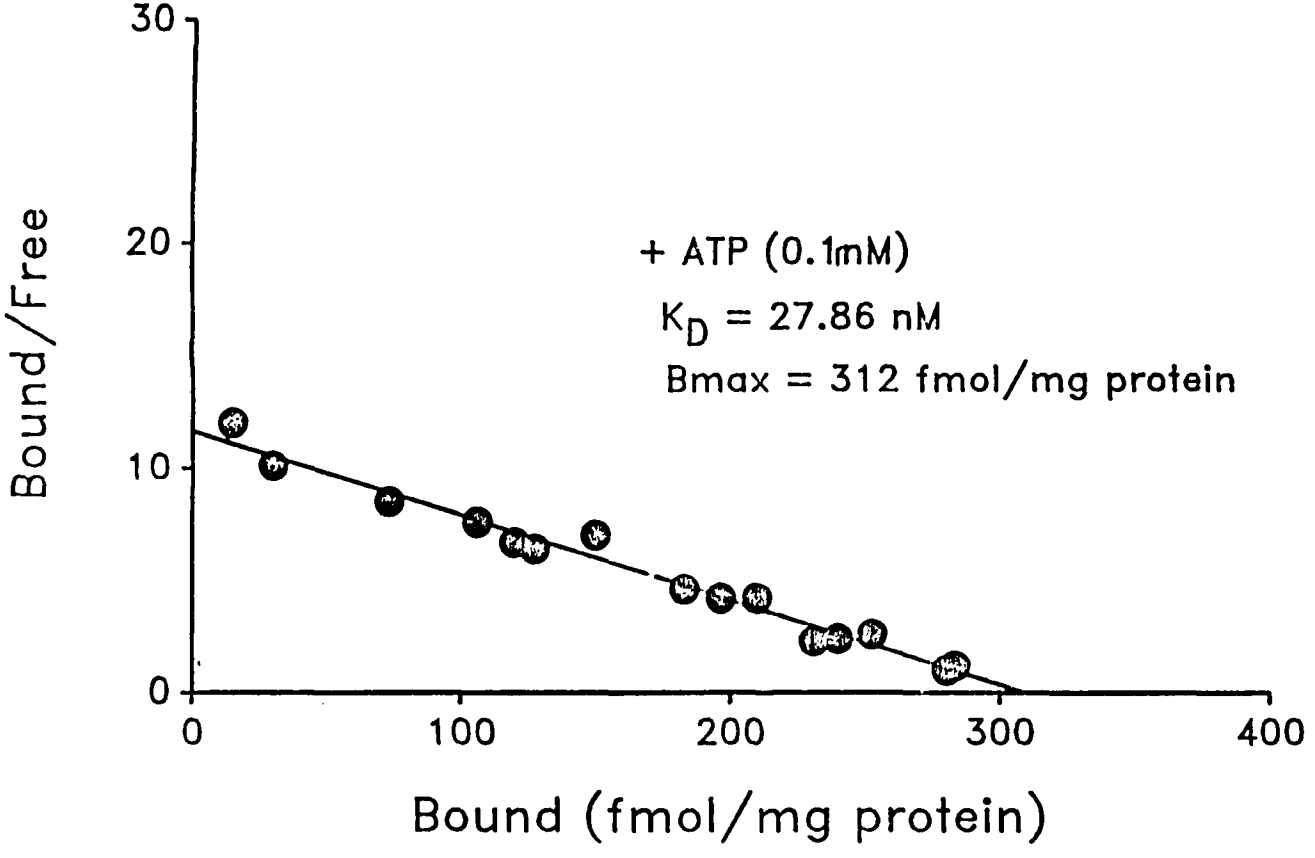

30
20
10
0
Bound/Free
+ ATP (0.1mM)
K_D = 27.86 nM
Bmax = 312 fmol/mg protein
0
100
200
300
400
Bound (fmol/mg protein)

FIGURE 13

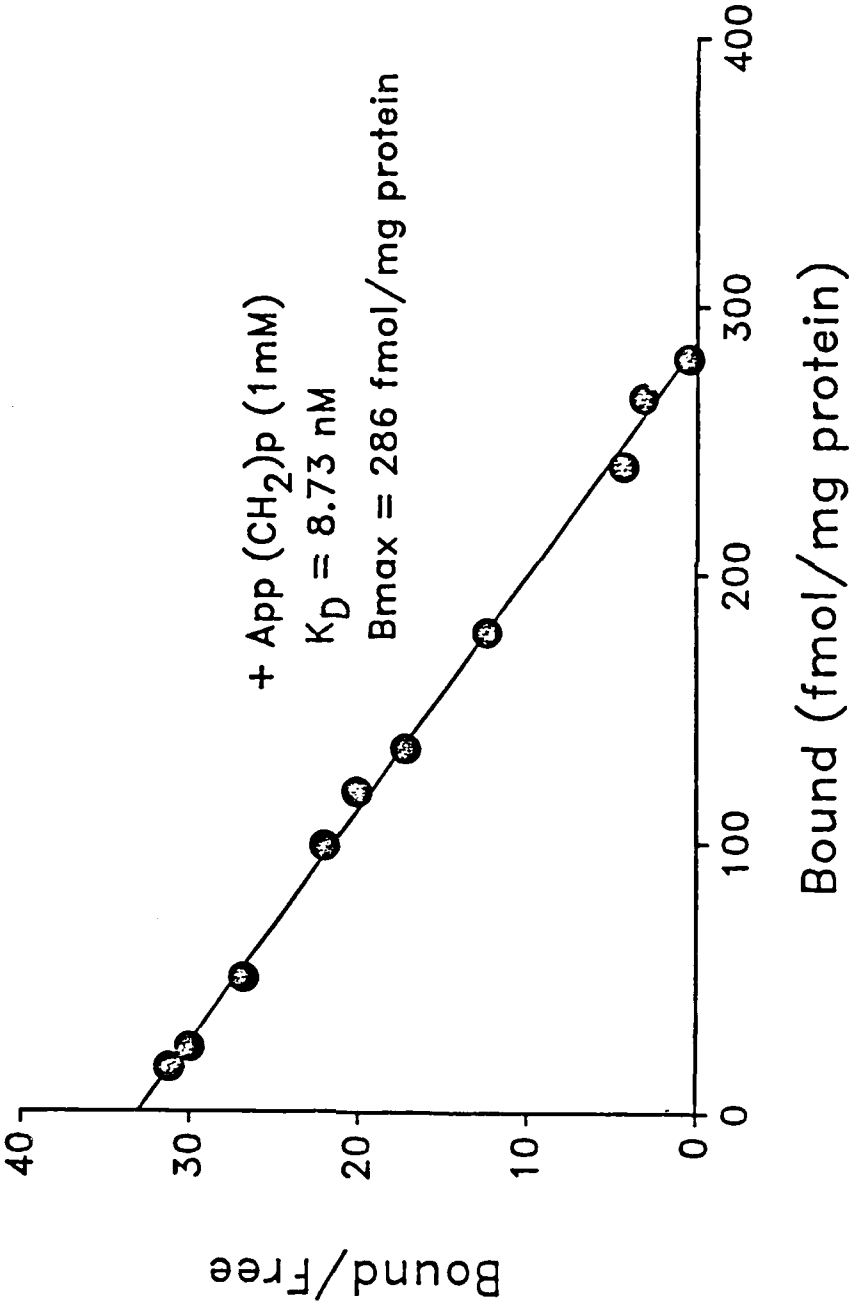

+ App $(CH_2)p$ (1mM)
K_D = 8.73 nM
Bmax = 286 fmol/mg protein
Bound/Free
Bound (fmol/mg protein)
0
10
20
30
40
0
100
200
300
400

FIGURE 14

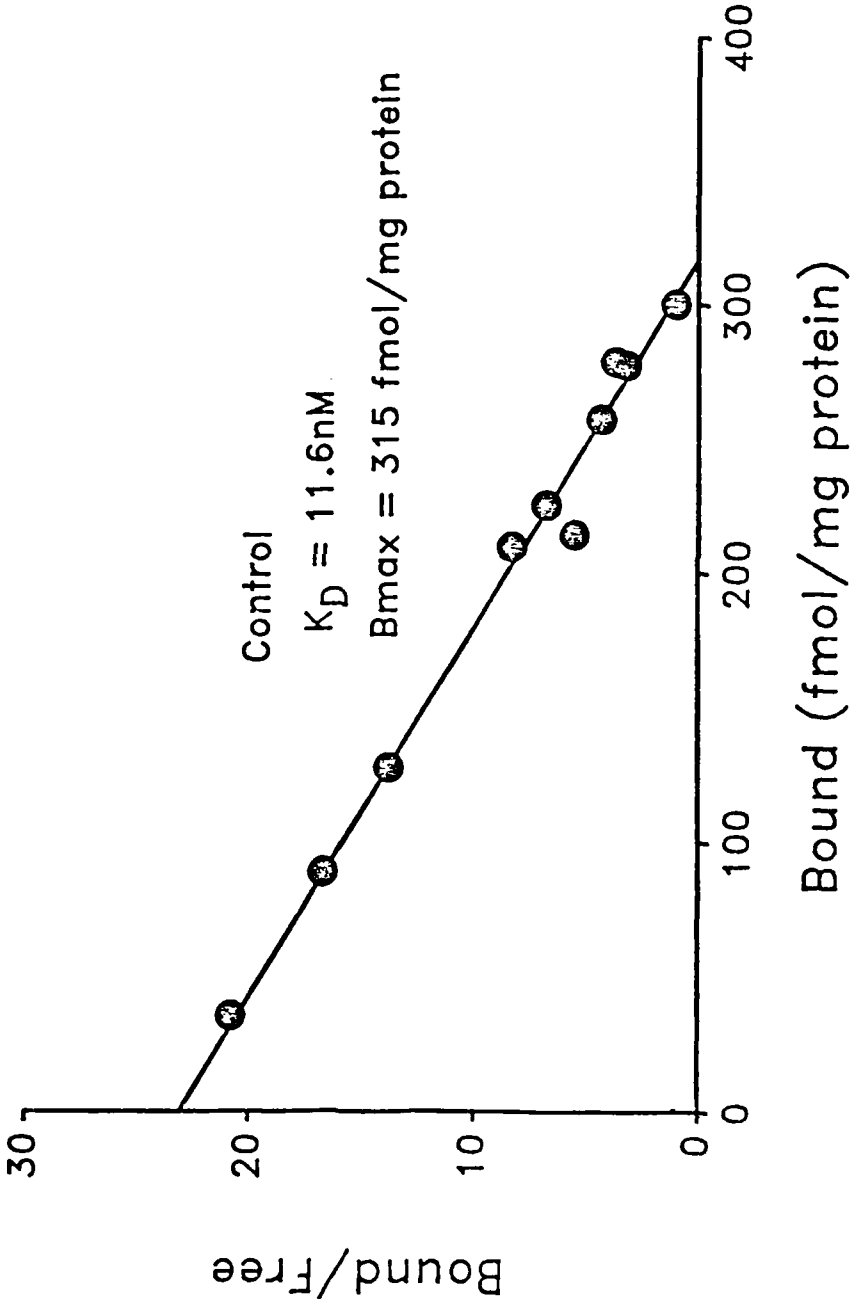

Control
K_D = 11.6nM
Bmax = 315 fmol/mg protein
Bound/Free
Bound (fmol/mg protein)
30
20
10
0
0
100
200
300
400

FIGURE 15

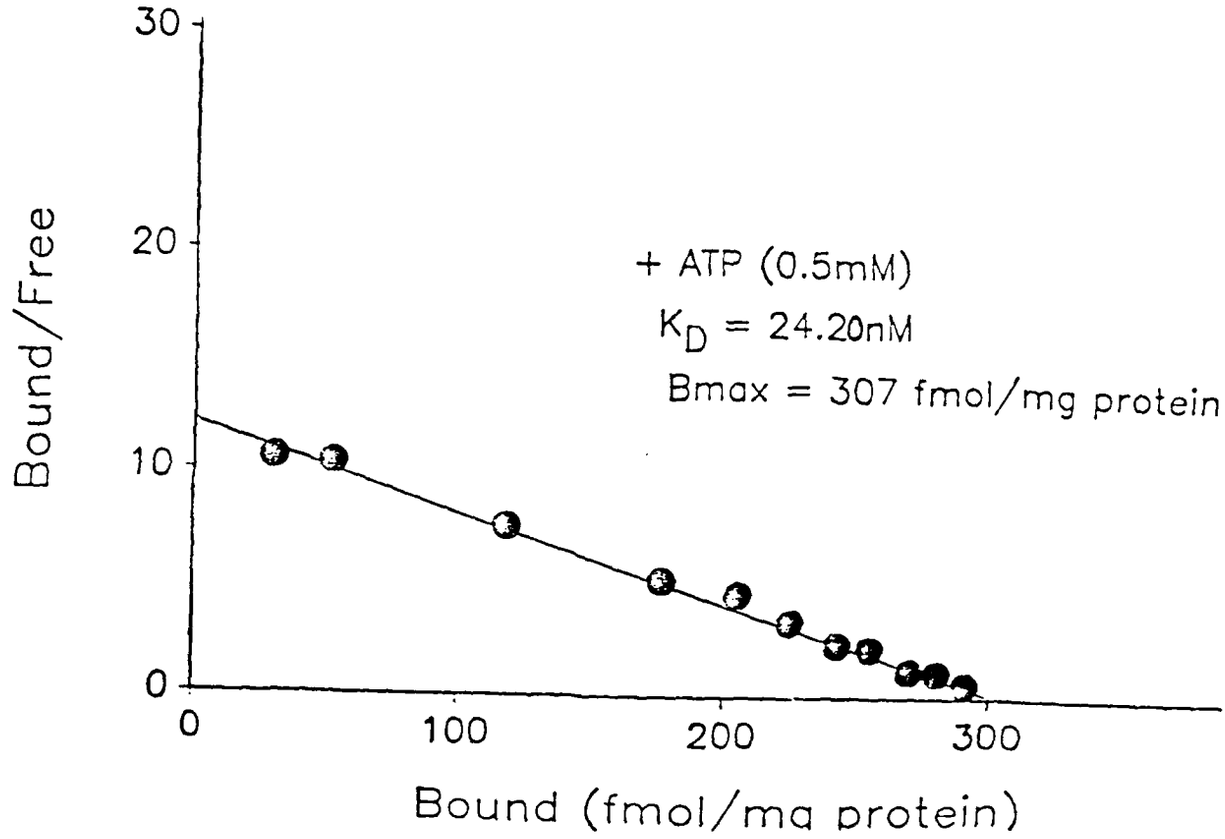

30
20
10
0
Bound/Free
+ ATP (0.5mM)
K_D = 24.20nM
Bmax = 307 fmol/mg protein
0
100
200
300
Bound (fmol/mg protein)

FIGURE 16

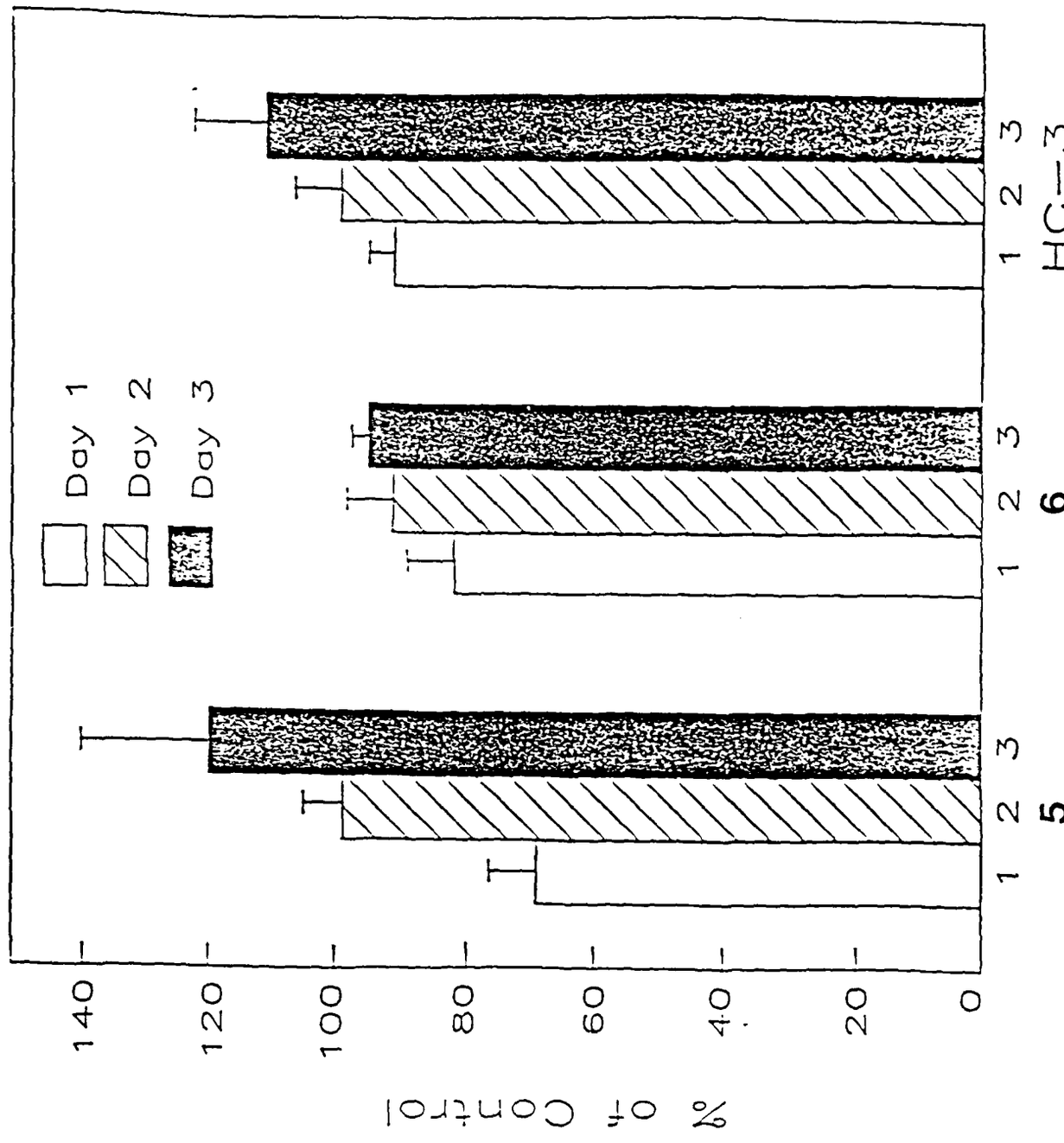
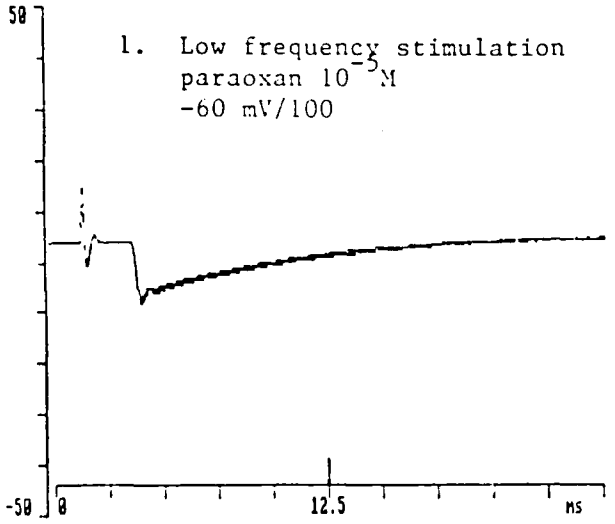
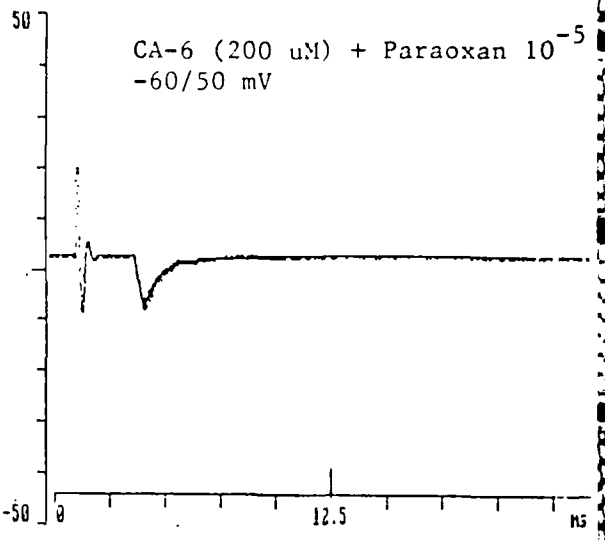
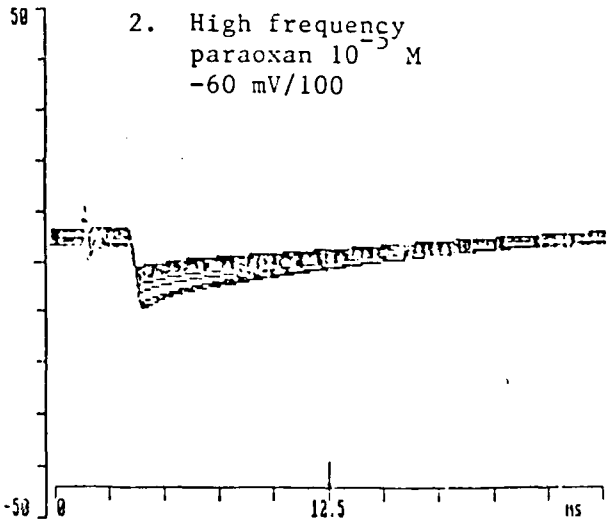

% of Control
140
120
100
80
60
40
20
0
Day 1
Day 2
Day 3
1 2 3
5
1 2 3
6
1 2 3
HC—3

FIGURE 17


50
1. Low frequency stimulation
paraoxan 10^{-5} M
-60 mV/100
-50
0
12.5
ms


50
CA-6 (200 uM) + Paraoxan 10^{-5}
-60/50 mV
-50
0
12.5
ms


50
2. High frequency
paraoxan 10^{-5} M
-60 mV/100
-50
0
12.5
ms

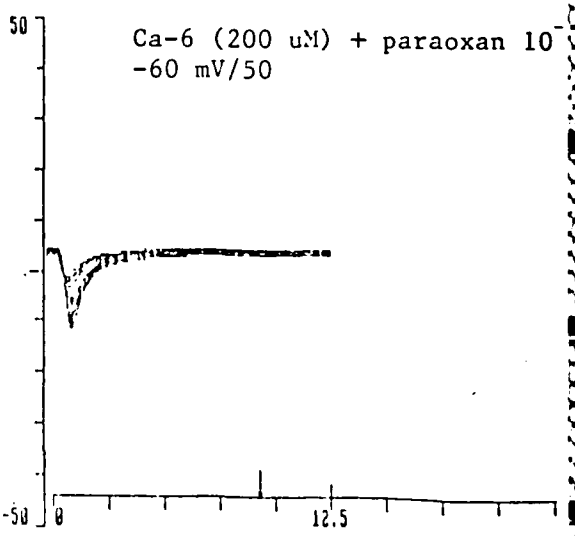
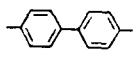
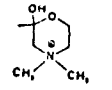
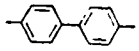
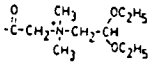
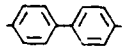
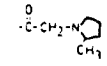
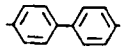
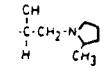
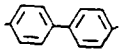
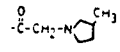
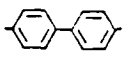
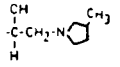
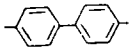
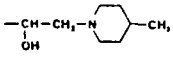
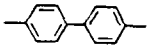
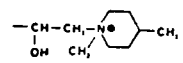
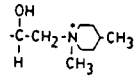
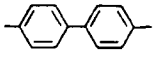
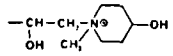

50
Ca-6 (200 uM) + paraoxan 10^{-}
-60 mV/50
-50
0
12.5

Table 1

Structure of Compounds

R'-R-R'

Compound	R	R'
HC-3		
DMAE		
1		
2		
3		
4		
5		
6		
7		
8		
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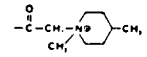
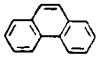
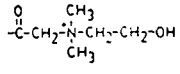
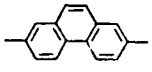
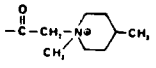
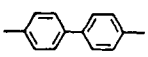
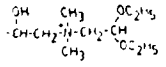
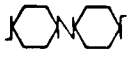
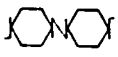
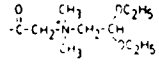
10		
11		
12		
13		
14		
15		

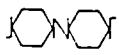
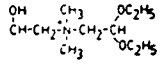
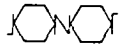
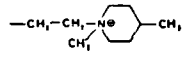
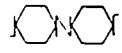
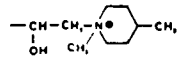
Compound	R	R'
16		
17		
18		
19		
20		
21		
22	$(CH_2)_7$	
23	$(CH_2)_6$	
24		
25		
26		
27		
28		
29		
30		
31		

Table 2

Inhibition of neuromuscular transmission in rabbits

Compound	ID_{50} (umol/kg)	95% Confidence limits	Reversible by choline
HC-3	0.007	0.006-0.01	+++
DMAE	0.058	0.03-0.1	+
5	4.02	3.2-5.0	++
6	0.003	0.002-0.006	+
7	0.05	0.3-0.07	+
8	0.98	0.6-1.7	+
9	0.24	0.02-0.03	+
10	0.45	0.04-0.053	+++
11	0.028	0.015-0.021	+++
12	0.023	0.022-0.026	0
13	0.98	0.8-1.2	+++
14	0.03	0.02-0.05	+
15	0.39	0.3-0.05	+
16	0.06	0.05-0.08	+
17	0.003	0.002-0.004	+
18	0.008	0.005-0.001	+++

+ or ++ = inhibition partially antagonized.
+++ = inhibition completely antagonized.

Table 3

Antagonism by compound DMAE and analogs of miniature end-plate current (mepc) induced by paraoxon or diisopropylfluorophosphate

Compound	Antagonism of mepc at frog neuromuscular junction
DMAE	++++
13	++++
15	+
16	+
19	ND[1]

[1] ND = not done

Table 4

Effect of DMAE and analogs on miniature end-plate currents in frog neuromuscular junction (voltage clamped at -80mv)

Compound	Peak mepc amplitude ED50 μM (95%CI)	Decay time constant ED50 μM(95%CI)
DMAE	57.3 (47.6-75.0)	32.9 (28.0-38.8)
13	72.4	46.9
16	128.8	NA*
19	NA*	NA*

*50% blockade is not observed with concentrations up to 160 μM.

Table 5

Toxicity data and antagonistic activity of compounds against paraoxon in mice

Compound	LD50 µmol/kg (95% CL)	Efficacy against paraoxon
Physostigmine	1.2 (0.75-1.39)	++++
Pyridostigmine	10.3 (3.3-14.2)	++++
Paraoxon (S.C.)	2.01 (1.62-2.38)	---
Paraoxon (S.C.)+ IM atropine (11.2 mg/kg)	5.89 (4.58-8.13)	+++++
HC-3	0.17 (0.1-0.3)	none
DMAE	0.97 (0.8-1.7)	++
13	7.39 (-0.8-9.2)	+++
15	8.4 (-23-13)	+++
16	0.6 (0.4-0.9)	+
17	0.29 (0.2-0.5)	none
20	0.48 (0.4-0.6)	none
21	1.36 (1.2-1.7)	none
22	3.6 (1.9-5.5)	+
23	1.3 (1.1-1.8)	none
24	0.14 (0.1-0.2)	none
25	4,183.3 (3900-4300)	++++
26	17.6 (15-19)	++++
27	7.7 (5.5-10.3)	none
28	1.5 (1.2-2.5)	+
29	0.26 (0.21-0.39)	+
30	0.9 (0.75-1.04)	none
31	6.7 (4.8-35.5)	none

++++ Among most active agents known.

Table 6

Inhibition of choline transport in synaptosomes
by two series of HC-3 analogs

Compound	IC50 nM
Hemiacetal group	
HC-3	18.3 ± 1.3
14	6.1 ± 0.8
32	6.2 ± 1.1
33	8.7 ± 1.3
Piperidine derivatives	
6	3.6 ± 0.2
7	3.3 ± 1.0
10	2.3 ± 0.3
12	1.0 ± 0.06
17	2.0 ± 0.1
18	1.4 ± 0.2
25	>1000
26	not done

Table 7

Effect of 5, 6 and HC-3 on choline uptake in the presence and absence of sodium ion in neuroblastoma cells

Conditions	Control (pmol/mg Protein)	5	6	HC-3
		% of Control		
1 μM Choline				
Sodium	310 ± 7.5	50.2 ± 5.1	37.1 ± 1.9	57.5 ± 2.9
Cesium	56.1 ± 2.8	65.5 ± 3.6	100.3 ± 4.9	107.3 ± 9.0
5 μM Choline				
Sodium	953.4 ± 41.0	59.6 ± 5.6	46.0 ± 5.3	74.1 ± 5.0
Cesium	235.1 ± 11.9	73.8 ± 2.7	87.4 ± 5.4	99.3 ± 9.6

The cells were pre-incubated in the presence or absence of sodium ions for 10 minutes. Choline was absent from pre-incubation buffer. The cells were then incubated in the presence of each experimetnal drug plus trace labeled choline (1 or 5 μM) for a five minute uptake period in the presence or absence of sodium. Compound 5 was at a concentration of 25 μM; compounds 6 and HC-3 were at 45 μM. Each value is expressed as mean ± S.E.M. and is the average of 6 separate determinations.

Table 8

Kinetic parameters of choline uptake in the presence of 5, 6 and HC-3 in neuroblastoma cells

Compound	K_m	V_{max}
Control	9.66 ± 0.627	69.83 ± 21.84
5	58.91 ± 11.74	180.32 ± 32.0
6	33.20 ± 8.20	95.30 ± 16.7
HC-3	13.60 ± 2.89	76.90 ± 14.89

The cells were pre-incubated in the presence of the compound in buffer for 15 minutes. Concentrations of the compounds used were 25 μM of 5 and 45 μM of 6 and HC-3. Uptake period was 5 minutes, with choline concentrations ranging from 0.1-10 μM. K_m and V_{max} values were calculated from the intercepts of double-reciprocal plots fitted by a weighted least-squares fit of the data points. The kinetic parameters are the means ± S.E.M. of 5 separate determinations. The units for K_m and V_{max}, respectively, are μM and pmol/min/mg protein.

Table 9

Effect of compounds 5 and 6 on choline incorporation into lipid in neuroblastoma cells over 24 hour incubation period

Length of Incubation (Hours)	Control	5	6
3	281.7 ± 8.87	231.0 ± 5.28	170.2 ± 11.71
6	651.1 ± 96.33	654.4 ± 71.22	498.1 ± 86.57
12	1348.2 ± 95.02	940.0 ± 124.72	905.9 ± 68.45
24	1987.0 ± 31.51	2145.4 ± 121.40	1450.4 ± 156.30

Cells were incubated for the length of time indicated with 25 μM 5 or 45 μM 6. Lipids were extracted with 2:1 chloroform: methanol and acid saline. Each value is expressed as the mean ± S.E.M. and is the result of 3 separate determination, units of the values are pmol choline/mg protein.

Table 10

Antagonism of acetylcholine-induced contractions of isolated rectus muscle of rana pipens (n=3)

Compound	ID50 μM (95% CL)	Relative potency (95% CL)
DMAE	14.3 (12.1-16.9)	1.0
13	8.7 (5.3-13.3)	1.6 (1.1-2.3)
15	10.8 (8.5-15.1)	1.4 (1.0-1.8)
16	4.3 (2.8-5.5)	2.5 (1.9-3.6)

Compound 19 did not reach 50% inhib. up to 324.2 μM.

Table 11

Antagonism of nicotine-induced positive chronotropic responses using isolated atria of guinea pigs (n=3)

Compound	ID50 μM (95% CL)	Relative potency (95% CL)
DMAE	0.064 (0.02-0.4)	1.0
1	2.01 (0.7-10)	0.045 (0.02-0.2)
2	0.8 (0.2-11)	0.21 (0.03-1.6)
3	5.1 (1.8-15)	0.017 (0.005-0.07)
13	0.037 (0.01-0.1)	1.31 (0.8-8.0)
15	0.064 (0.03-0.13)	1.26 (0.6-3.0)
16	0.081 (0.001-0.1)	1.14 (0.5-3.1)
19	0.025 (0.005-0.08)	3.54 (0.8-23.7
22	0.25 (0.1-0.8)	0.43 (0.16-1.2)

Table 12

Inhibition of choline uptake by isomers of compounds 5 and 6 in neuroblastoma cells[1]

Compound	ID50 μM (95% CL)
5	
racemic	28.3 (24-9-32.7)
d-isomer	17.1 (13.6-20.2)
l-isomer	23.8 (19.2-28.9)
meso-form	26.1 (21.1-33.1)
6	
racemic	46.8 (37.7-66.0)
d-isomer	40.2 (34.2-49.6)
l-isomer	23.8 (19.2-28.9)
meso-form	26.1 (21.1-33.1)

[1] N=5 for each compound.

Table 13

Inhibition of rabbit sciatic
nerve-gastrocnemius muscle preparation

Compound	ID50-umol/kg (95% CL)
5	
racemic	2.6 (2-4)
meso-form	1.6 (0.8-3)
d-isomer	1.6 (0.6-2.9)
l-isomer	3.5 (1.9-21.9)
6	
racemic	0.003 (0.002-0.006)
meso-form	0.006 (0.005-0.009)
d-isomer	0.012 (0.009-0.019)
l-isomer	0.006 (0.004-0.009)
13	0.98 (0.8-1.2)
15	0.39 (0.3-0.5)
16	0.06 (0.05-0.08)
19	1.06 (0.7-2.0)
38	0.31 (0.2-0.7)
39	0.30 (01-4.4)
HC-3	0.007 (0.006-0.011)

Table 14

Inhibition of choline transport by isomers of 5 and 6 in synaptosomes

Compound	Synaptosomes IC-50 nM (mean ± SEM)
Racemic 5	31.76 ± 5.88
d-isomer	41.38 ± 8.82
l-isomer	31.54 ± 1.69
meso-form	35.93 ± 8.12
HC-3	18.28 ± 1.34
Racemic 6	3.55 ± 0.17
d-isomer	3.50 ± 0.04
l-isomer	2.51 ± 0.44
meso-form	2.56 ± 0.46

Acknowledgments

We wish to thank the Army's Drug Development Program for support of our research. Without your fiscal support this research would not have been done. We wish to thank Dr. H.A. Musallam and Larrel Harris for their encouragement and guidance.

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